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(54) Title: APPLICATIONS FOR REGULATORY REGION OF NOS ISOFORMS

(57) Abstract

The regulatory peptides for constitutive nitric oxide synthase enzymes, and a peptide specific to inducible nitric oxide synthase, as well as derivatives of the peptides, homologous peptides, nucleic acids encoding the peptides, derivatives, and homologous peptides, and antibodies to the peptides, derivatives, and homologous peptides, are disclosed. The peptides, derivatives, homologous peptides, antibodies, and nucleic acids, as well as peptidomimetics, can be used in methods of modulating the activity of nitric oxide synthase enzymes, and also in methods of treating diseases or conditions modulated by production of nitric oxide by nitric oxide synthases. Assays for identifying agents which modulate the activity of the nitric oxide synthase enzymes are also described.

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# APPLICATIONS FOR REGULATORY REGION OF NOS ISOFORMS

## RELATED APPLICATION

This application is a Continuation-in-Part of U.S. Serial No. 08/679,006, filed July 12, 1996, the entire teachings of which are incorporated herein by reference.

## BACKGROUND OF THE INVENTION

Nitric oxide (NO), a small molecule which is highly toxic at moderate concentrations, is a key messenger in mammalian physiology. NO is produced in humans by three related enzymes which comprise the nitric oxide synthase (NOS) family.

Endothelial NOS (ENOS) produces NO which controls vascular tone (hence blood pressure), dilates the airways, and controls numerous processes dependent on local dilation of blood vessels (such as gas exchange in lungs, penile erection, and renal function). Brain or neuronal NOS (BNOS or NNOS) produces NO which functions as a neurotransmitter. It controls peristalsis in the gut, and is implicated in neural potentiation and brain development. NNOS and ENOS are constitutive enzymes controlled by intracellular calcium and the regulatory protein calmodulin (CAM). When the level of calcium in the cell rises, NNOS and ENOS bind calmodulin and are turned on to start NO production.

A third, inducible NOS, immune NOS or macrophage NOS (INOS or MNOS), is synthesized by the immune system in response to an immune challenge. Upon induced expression, this enzyme is always active; it has a calmodulin binding site, but binds calmodulin tightly even at low calcium levels. INOS produces orders of magnitude more NO than

other NO synthases. This NO level is cytotoxic to tumor cells, bacteria, and other pathogenic organisms.

While INOS thus appears to be an important component of immune response, its activity is highly toxic as well. Excess production of NO by INOS can be responsible for toxic shock syndrome, septic shock, and killing of islet cells in diabetes. Excess NO production by INOS has also been implicated in a wide range of other autoimmune conditions, including arthritis and other inflammatory conditions.

Thus, it is of critical importance to learn to control NO synthesis by one NOS, without interfering with the activity of other NO synthases. Currently, inhibitors of INOS also inhibit NNOS and ENOS.

## SUMMARY OF THE INVENTION

The current invention concerns recently discovered intrinsic control site elements of constitutive nitric oxide synthases. These intrinsic control site elements, referred to as "regulatory peptides," include the regulatory peptide of endothelial nitric oxide synthase (ENOS), MSGPYNSSPRPEQHKSYKIRFNSVSCSDPLVSSWRRKRKESSNTD (SEQ. ID. NO. 1); the regulatory peptide of neuronal nitric oxide synthase (NNOS) MRHPNSVQEERKSYKVRFNSVSSYSDSRKSSGDGPDLLRDNFE (SEQ. ID. NO. 2); a polypeptide specific to inducible nitric acid synthase (INOS), KKSLFMLRELNHTFRY (SEQ. ID. NO. 3). Based on this discovery, methods are now available to identify agents that modulate (activate or inhibit) NOS activity, as well as the agents themselves. Agents include agents that inhibit NOS activity by blocking calmodulin activation of the NOS enzyme; agents that inhibit NOS activity by blocking electron transfer from NADPH to an active site in NOS; agents that activate a constitutive NOS enzyme by antagonizing autoinhibition of a regulatory region of the NOS enzyme; and agents that modulate NOS

activity by interacting with the regulatory peptide or spatially adjacent control regions. The agents include the peptides described above, as well as derivatives of these peptides, and homologous peptides. Homologous peptides include substantially isolated peptides having an array of at least two positively charged amino acids, and an amino acid sequence of at least about 60% homology, or about 67% homology, or about 80% homology, or about 90% homology, to the amino acid sequence of the ENOS regulatory peptide; peptides having an amino acid sequence of at least about 60% homology, or about 67% homology, or about 80% homology, or about 90% homology, to the amino acid sequence of the NNOS regulatory peptide; peptides having an amino acid sequence of at least about 60% homology, or about 67% homology, or about 80% homology, or about 90% homology, to the amino acid sequence of the INOS-specific peptide; and peptides having an amino acid sequence of at least about 60% homology, or about 67% homology, or about 80% homology, or about 90% homology, to the amino acid sequence of the negatively charged loops of the NOS enzymes. The invention further concerns nucleic acids encoding the peptides, derivatives, and homologous peptides; fusions of peptides with proteins or other macromolecules (e.g., polysaccharides); peptidomimetics of the peptides, derivatives, and homologous peptides; and antibodies (either monoclonal or polyclonal antibodies, or fragments thereof) to the peptides, derivatives, and homologous peptides.

The agents can be used to modulate the activity of NOS enzymes. Preferably, the agents modulate the activity of one NOS enzyme, but do not substantially affect the activity of the other NOS enzymes. Such agents can be used to treat diseases or conditions mediated by production of nitric oxide by inducible nitric oxide synthase, such as toxic shock, septic shock, autoimmune diseases,

inflammatory conditions, and diabetes. The agents can also be used to treat diseases or conditions mediated by production of nitric oxide by a constitutive NOS enzyme, such as hypertension, diabetes, or AIDS-related dementia.

Thus, as a result of the discovery described herein, it is now possible, for the first time, to design and/or isolate isoform-specific inhibitors, and also isoform-specific activators, of the different NOS isoforms. This overcomes the limitations of earlier methods, which restricted drug discovery to analogs of substrates, such as arginine, or cofactors, such as tetrahydrobiopterin. Furthermore, the current discovery allows identification of activators of NOS enzymes, which was previously impossible.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 demonstrates the alignment of a selected set of NOS, cytochrome P450 reductase (CPR), and bacterial flavodoxin sequences, illustrating the conservation of regions involved in FMN binding.

Figures 2A-2B provides evidence of transient activation of ENOS and NNOS by antibodies to the autoinhibitory sequences. Figure 2A shows that addition of antibody to a reaction mixture containing ENOS and arginine but not CAM produces a ~1 minute burst of NADPH consumption, followed by a phase of less marked activation. Figure 2B shows that addition of fresh enzyme (NNOS is shown) to a reaction mixture which has already passed through its rapid phase leads to a second burst of NADPH consumption. Addition of fresh antibody (not shown) had little or no effect, indicating that the end of the rapid phase was due to loss of native enzyme. We have obtained similar results with antibodies to several versions of the synthetic control site analogs, and with both ENOS and NNOS.

Figures 2C-2D confirms the activation of electron transfer by the antibody, and indicates the probable reason for the transient nature of activation. Addition of antibody to a reaction mixture containing CO and NADPH produced the spectrum shown at top, with a ~420 nm Soret band visible on the side of the UV absorbance of NADPH. Ferroheme-CO binding indicates that intramolecular electron transfer (the CAM controlled step in physiological conditions) has occurred. Dithionite addition to a similar reaction mixture produced the spectrum with the Soret band just below 450 nm. The difference spectra in the lower panel show these bands clearly. Again, we have obtained similar results with antibodies to several different synthetic analogs, and with both ENOS and NNOS. The Soret band formed by dithionite addition in the presence of CO is characteristic of the ferrous CO adduct of a heme with a thiolate axial ligand; an identical complex can be formed by addition of CAM in the present of NADPH (not shown). The 420 nm band is also due to the formation of a ferrous CO complex, confirming the activation of electron transfer, but indicates that the axial thiolate ligand of the native heme site has been lost. This is closely analogous to the conversion of P450 enzymes to the inactive P420 form.

The foregoing and other objects, features and advantages of the invention will be apparent from the following more particular description of preferred embodiments of the invention, as illustrated in the accompanying drawings in which like reference characters refer to the same parts throughout the different views. The drawings are not necessarily to scale, emphasis instead being placed upon illustrating the principles of the invention.

DETAILED DESCRIPTION OF THE INVENTION

The current invention pertains to the discovery of the existence and identity of regulatory peptides of constitutive nitric oxide synthase (NOS) enzymes. As described in the Examples below, Applicant has identified the regulatory peptide of constitutive NOS enzymes as an intrinsic polypeptide insert in the flavin mononucleotide (FMN) binding domain of endothelial nitric oxide synthase (ENOS), MSGPYNSSPRPEQHKSYKIRFNSVSCSDPLVSSWRRKRKESSNTD (SEQ. ID. NO. 1) and in brain or neuronal nitric oxide synthase (NNOS) MRHPNSVQEERKSYKVRFNSVSSYSDSRKSSGDGPDLLRDNFE (SEQ. ID. NO. 2). Inducible nitric oxide synthase (INOS) lacks a similar polypeptide insert; instead, INOS has an INOS-specific region (the "INOS-specific polypeptide"), amino acids 600-615 of INOS (SEQ. ID. NO. 3), a short loop which is split and greatly extended by the introduction of the regulatory peptide (also referred to herein as the intrinsic peptide) in ENOS and NNOS. Applicant has also identified a core region of the ENOS binding domain: the array of positively charged amino acids, RRKRK (SEQ ID NO. 10), within the ENOS binding domain, alters activity of the enzyme.

As a result of the discovery, methods are now available for isolating or identifying an entirely new class of NOS isoform-specific inhibitors or activators. As disclosed herein, it is now possible to design and/or screen for agents which modulate (either inhibit or activate) a specific isoform of NOS, without altering the activity of other isoforms. The invention includes both the methods by which agents can be isolated or identified, as well as those agents so isolated and identified.

Agents of the invention include agents that modulate, and particularly inhibit, nitric oxide synthase activity by, for example, blocking activation of the NOS by calmodulin. The agents also include agents that modulate,

and particularly activate, a constitutive NOS by, for example, antagonizing or interfering with autoinhibition of the enzyme by a regulatory region of the enzyme, as described herein. The agents also include agents which bind to sites spatially adjacent to the calmodulin binding site of a constitutive NOS enzyme. The agents further include agents that inhibit NOS by blocking electron transfer from NADPH to an active site in NOS. Representative agents include certain substantially isolated peptides, including the regulatory peptides of ENOS and NNOS, the INOS-specific peptide, the negatively charged loops of INOS, ENOS and NNOS, as well as derivatives of the peptides and homologous peptides. Nucleic acids encoding the peptides, derivatives and homologous peptides are also available, as are antibodies specific to the peptides, their derivatives and homologs. Agents which bind to these peptides, as well as agents that bind to the control regions of the NOS isoforms, and agents which bind to functional groups within the INOS-specific peptide, are also agents of the invention. In addition, methods of altering the activity of ENOS, NNOS, or INOS; methods of treating diseases mediated by production of nitric oxide; methods of treating conditions mediated by ENOS; and methods of treating conditions mediated by NNOS, are available. The methods include use or administration of the agents described herein.

The discussion below presents screening methods for agents that modulate NOS enzymes; screening methods for agents that inhibit INOS; screening methods for agents that activate constitutive NOS enzymes; preparation and design of agents that bind to control sites for NOS isoforms; peptides, probes, nucleic acids, and antibodies of the invention; and uses of agents, peptides, and antibodies of the invention, including methods of treatment of certain

diseases or conditions associated with NO production in mammals, including humans.

Nitric Oxide Synthases: Structure, Function and Control

Bredt, Snyder and coworkers (Bredt, D.S. *et al.*, *Nature 351*:714-718 (1991) and Bredt, D.S. *et al.*, *J. Biol. Chem. 267*:10976-10981 (1992)) showed that the C terminal regions of all three NOS isoforms contained sequences homologous to cytochrome P450 NADPH reductase (P450, CPNR) and proposed locations for the FMN, FAD, and NADPH binding sites. Sheta, E.A. *et al.*, *J. Biol. Chem. 269(21)*:15147-15153 (1994) cleaved NNOS into two fragments during limited trypsinolysis; cleavage occurred within the CAM binding sequence. The C terminal (reductase) fragment contained the regions identified by Bredt and Snyder, while the N terminal (oxygenase) fragment contained the arginine binding site, tetrahydrobiopterin, and heme. The expression of constructs corresponding to these regions in NOS, INOS and ENOS, as well as NNOS holoenzyme, in *E. coli* has also been described by Masters, B.S. *et al.*, *Faseb J 10(5)*:552-558 (1996); McMillan, K. and Masters, B.S., *Biochemistry 34(11)*:2686-2693 (1995); Nishimura, J.S. *et al.*, *Biochem Biophys Res Commun 210(2)*:288-294 (1995); McMillan and Masters 1995; Nishimura, Martasek *et al.* 1995; Masters, McMillan *et al.* 1996; see also Matsuoka, Stuehr *et al.* 1994; Ghosh, Abusoud *et al.* 1995; Ghosh, Abusoud *et al.* 1996).

The oxygenase domains of ENOS and INOS extend from the N terminal to the CAM binding site. In NNOS, an additional N terminal sequence of about 200 amino acid residues is homologous to sequences in the N terminal region of DLG1 (in *Drosophila melanogaster*) and PSD9, a rat neuronal protein involved in control of cell proliferation. Both proteins appear to be involved in signal recognition (Cho, K.-O. *et al.*, *Neuron 9*:929-924 (1992)). Brenman and

coworkers (Brenman, J.E. *et al.*, *Cell 82*:743-752 (1995)) recently reported that this region functions as a protein recognition site. While both DLG1 and PSD9 are tyrosine kinases, the kinase active site does appear to be present in NOS.

Cys 415 was identified as the axial heme ligand of bovine NNOS by site directed mutagenesis (McMillan, K. and Masters, B.S., *Biochemistry 34(11)*:3686-3693 (1995)), and corresponding cysteinyl residues are axial ligands in the other isoforms (Chen, P.-F. *et al.*, *J. Biol. Chem. 269*:25062-25066 (1994)) as previously proposed (McMillan, K. *et al.*, *Proc. Natl. Acad. Sci. USA 89(23)*:11141-11145 (1992)). The NOS oxygenase domains show no extensive similarity to P450. Some similarity between pterin binding proteins and NOS has been reported (Nishimura, J.S. *et al.*, *Biochem. Biophys. Res. Commun. 210(2)*:288-294 (1995)). Constructs based on regions of apparent similarity can be expressed, but neither these constructs or constructs including the remainder of the oxygenase domain can be shown to bind tetrahydrobiopterin. The independently expressed construct reported by Nishimura *et al.* (residues 558-721 in NNOS) does bind N nitroarginine, however, indicating that it contains a significant part of the substrate binding site.

Several lines of evidence indicate that the binding sites for heme, pterin, and arginine are in close contact. Clearly, the oxygen adduct of the ferroheme must be in close proximity to the arginine ε amino group to initiate the hydroxylation reaction. Arginine competes with bulky heme ligands (imidazole) and influence the kinetics of the binding of diatomic ligands; arginine and arginine analogs influence both the spin state of the ferriheme and the ligation geometry of the high spin ferriheme. Tetrahydrobiopterin influences the binding of arginine and arginine analogs and more subtly affects the heme ligation

geometry. The presence of strong interactions between these sites complicates the unambiguous determination of their locations in the sequence using site directed methods or constructs corresponding to limited regions of sequence.

An axial ligand of ENOS and INOS is located in a conserved region with the consensus sequence KXAWRNAXRCZGRIQWXXLQVFDARD (SEQ ID NO: 36), where Z is valine or isoleucine. In addition to the axial ligand, there are five conserved basic residues in twenty-five residues. There are only five conserved basic residues each in the preceding and following hundred residue segments, even if substitutions were allowed within the set of histidine, lysine and arginine. In other heme proteins, it is common for basic residues close to an axial ligand in the sequence to interact with a propionate group on the porphyrin periphery. In the known P450 structures, one such propionate ligand is provided by the second residue before the axial ligand, and a second by about ~50 residues before the axial ligand. Therefore, at least one of the basic residues in the sequence given above is likely to be a propionate ligand, and it is not impossible that several such ligands are contributed by the sequence. The short sequence around the axial ligand can function as a heme binding cassette when inserted in a region with appropriate structural determinants.

NOS is active only as a dimer (Abu-Soud, H.M. *et al.*, *Biochemistry 34*:11167-11175 (1995)). Independently expressed heme protein, but not flavoprotein, is dimeric, indicating that the intermonomer interactions are primarily between the oxygenase regions (McMillan, K., and Masters, B.S., *Biochemistry 34(11)*:3686-3693 (1995)). In previously suggested 'head to tail' models, the reductase unit of one monomer reduces the oxygenase unit of the other (Schmidt, H.H.H. *et al.*, *Proc. Natl. Acad. Sci. USA 88*:365-369 (1991)).

The FMN binding modules of NOS isoforms are homologous to the corresponding region in CPNR and in turn to flavodoxins, a family of small FMN binding proteins. The INOS sequence closely corresponds to that of *Desulfovibrio* flavodoxin. A major insertion is present in ENOS and NNOS relative to flavodoxins and CPNR corresponding to the addition of an ~45 residue subdomain. Alignments including numerous additional sequences of similar FMN binding domains suggest that only CNOS sequences have such an insertion, which is absent in INOS. this insertion represents the most important difference between the CNOS and INOS sequences, and correlates with $Ca^{+2}$/CAM control.

All enzymes containing this module contain three regions involved in FMN binding (Porter, T.D., *Trends in Biological Sciences 16*:154-158 (1991)) extending from the CAM binding site to the C terminal. Bredt, D.S. *et al.*, *Nature 351*:714-718 (1991)) identified a region contributing several residues interacting the FMN, including a shielding aromatic. An additional region near the N terminal of the module forms a number of hydrogen bonds with the terminal phosphate group of FMN, and a third region contributes a second aromatic ring in contact with FMN as well as hydrogen bond partners.

Homology based models have been constructed using the Insight, Homology and Discover modules of the Biosym suite of molecular modeling software. The INOS FMN domain model structure closely corresponds to *Desulfovibrio vulgaris* flavodoxin. The domain consists of a five stranded parallel $\beta$ sheet with primary helical returns and the FMN binding site along one edge on a Rossman fold motif. The aromatic side chains of two residues (F587 and Y625 in INOS) are in contact with the isoalloxazine ring system. The structures of the corresponding domains of ENOS and NNOS are also similar; the primary difference is the insertion of a ~45 residue subdomain structure in the CNOS

sequences. In all NOS isoforms there are three regions which contribute to the binding of FMN; the initial region is involved in a network of hydrogen bonds with the terminal phosphate group, while the second and third regions provide aromatic side chains which interact with the isoalloxazine ring system as well as hydrogen bond partners. The proximity of the first FMN binding region to the CAM binding site allows the N terminal edge of the FMN binding domain to be specified with great confidence.

The FAD and NADPH binding domains of NOS can be modeled on the basis of homology with ferredoxin NADPH reductase (FNR). FNR consists of a five stranded parallel $\beta$ sheet domain and an eight stranded $\beta$ barrel.

Homology models of the FAD and NADPH binding domains of NNOS establish that the isoalloxazine ring of FAD lies against the outside of the $\beta$ barrel and interacts with a conserved aromatic residue inside the barrel. The adenine binding site is located on a loop connecting two barrel strands. A stacking interaction for the isoalloxazine ring is provided by the last residue in the sheet domain, which is otherwise devoted to NADPH binding.

The NADPH binding module is a five stranded antiparallel $\beta$ sheet with helical returns. NADPH is a dinucleotide; an adenine binding site, including a stacking aromatic residue, is located on an $\alpha>\beta$ loop. The nicotinamide moiety was not bound in any solved FNR structure, but can be modelled as stacked with the FAD isoalloxazine ring system.

In addition to the three cofactor binding domains and the short connecting linkers which join them, there are three other significant regions in the NOS reductase segment. The aromatic residue which provides a stacking interaction with FAD is the terminal residue in FNR, and the penultimate residue in NCPR. All three NOS isoforms extend significantly beyond the NADPH binding domain, which

ends with this residue. Homology modeling of the NADPH/FAD binding region indicates that the structure formed by the C terminal extension lies close to the cofactor binding sites.

Functional considerations suggest that the FMN binding domain is oriented so that the cofactor binding site occupies the edge facing the other cofactors. The structure formed by the NOS C terminal extension is thus well positioned to mediate interactions between domains. This is likely to include interactions with the oxygenase domains, since it is likely that, at least in the active state, the heme binding region will be in close proximity to the three reductase cofactors, particularly to the FMN which is probably its immediate reductant.

Interactions between the FMN and FAD binding domains are mediated by a subdomain, present as an insertion in the FAD binding domain between sequences forming two strands of the $\beta$ barrel. This subdomain is not present in FNR. It is large enough to form extensive regions of secondary structure.

The requirements of efficient electron transfer imply an orientation of the FMN binding domain in which the FMN faces the other cofactors. A subdomain is present as an insertion in the FAD binding domain in all proteins which contain both FNR and flavodoxin modules. It corresponds to an extended loop (with extensive secondary structure) between two strands of the $\beta$ barrel and mediates interactions between the FAD and FMN binding domains.

The primary site of oxygen chemistry in NOS is the heme; the arginine and tetrahydrobiopterin binding sites are also located in the N terminal half of the molecule. The reductase domains function in catalysis is limited to the delivery of reducing equivalents to the catalytic site. It is the delivery of electrons to the catalytic site, however, that is regulated by CAM binding. CAM binding has

been reported to affect electron transfer within the reductase domains as well as FMN/heme electron transfer (Abu-Soud, H.M. *et al.*, *Journal of Biological Chemistry* *269*:32050 (1994)).

Clearly, the FMN binding domain must be oriented so that the FMN is close to the isoalloxazine ring system of FAD in the adjacent domain. This places the CAM binding site, which is at the opposite edge of the $\beta$ sheet from FMN, in a location remote from the reductase cofactor binding sites. The C terminal extension regions of all three NOS isoforms are in a position to mediate Interdomain interactions, are also remote from the CAM binding site. These regions, and the subdomain of the FAD binding module, are of potential significance in stabilizing different aspects of the active conformation but are unlikely to be directly involved in control.

The organization of the reductase domains suggests that the CAM binding site is remote from the flavin binding sites. The C terminal extension regions of all three NOS isoforms are adjacent to the flavin sites and hence distant from the CAM binding site. None of these regions, or the subdomain of the FAD binding module, is likely to be directly involved in control.

The major insertion in the FMN binding domain is directly adjacent to the CAM binding site in our model structure, and the presence of this element correlates perfectly with CAM/$Ca^{+2}$ control. Silvagno *et al.* (Silvagno, F. *et al.*, *J. Biol. Chem.* *271*:11204-11208 (1996)) identified it as the locus of a tissue specific alternatively spliced form of NNOS. Lowe *et al.* (Lowe, P.N. *et al.*, *Biochem. J.* *314*:55-62 (1996)), in analyzing the tryptic cleavage fragments reported by Sheta *et al.* (Sheta, E.A. *et al.*, *J. Biol. Chem.* *269(21)*:15147-15153 (1994)), pointed out that one early cut site was located in this region, and speculated on a possible role in

intersubunit interactions. In particular, synthetic peptides homologous to the insertions discussed herein (particularly to the ENOS insertion) are inhibitors of CNOS activity and of CAM binding.

The requirements for efficient FMN/heme electron transfer place the heme binding site close to the flavin binding end of the FMN binding module. In P450 enzymes reduced by the homologous P450 reductase, electron transfer into the heme site from FMN takes place through the face of the P450 defined by the loops carrying the axial thiolate ligand. A similar arrangement can prevail in NOS; the opposite orientation would place the extended arginine binding site between the donor and acceptor. Structurally, the 'heme binding cassette' can be close to the FMN, with either the thiolate binding face of the heme or an edge adjacent to FMN. This end of the oxygenase region can be positioned in part by interactions with the extreme C terminal region of the reductase domains.

Since the CAM binding domain is attached to the other end of the FMN module, and since steric constraints suggest that it oriented away from the string of prosthetic groups which make up the electron transfer pathway, the oxygenase and reductase domains can be connected by a long 'strap' of polypeptide connecting the end of the CAM binding site with the oxygenase region, or utilizing the dimeric nature of the active enzyme.

NOS is active only as a dimer. It has been previously suggested from other evidence that the reductase domains of one monomer may reduce the oxygenase domains of the other. Furthermore, independently expressed heme protein, but not flavoprotein, is dimeric, indicating that the monomer/monomer interactions are primarily between the oxygenase regions. A considerable body of evidence has been amassed which suggests that dimerization and cofactor

binding are coupled, although the minimum cofactor requirement for dimerization is isoform dependent.

Each reductase unit possibly donates electrons to the oxygenase unit of the other monomer. It is the heme site of the other monomer which is in proximity to the FMN in the active state. Each reductase unit is joined through the CAM binding region to the oxygenase unit of the same monomer, but interacts across a wide molecular surface with the oxygenase unit of the other monomer. This molecular surface is formed by the junction of the FMN and FAD binding domains, and can include structures formed from the C terminal extension.

Interactions between the heme and pterin binding regions of the oxygenase units and the flavin binding regions of the reductase units probably mediate the linkage between cofactor binding and dimerization. The results of removing these interactions as a loss in the ability of the heme sites in heme protein constructs to maintain a narrow range of conformations in the absence of the reductase domains have been observed. In order to form close contacts between the oxygenase and reductase cofactor binding regions, dimerization is required. Models have explained this synergy by the binding of cofactors as bridging ligands between monomers. It is far more likely that cofactor binding and dimerization equilibria are linked by a requirement for similar restricted sets of conformations; in other words, both cofactor biding and dimerization require the cofactor region to be in 'the native state'.

Antibodies raised to these polypeptide inserts bind strongly to both ENOS and NNOS; homology between the two CNOS isoforms leads to cross reactivity. The effect of antibody binding on the ferriheme optical spectra have been studied. The Soret difference spectrum has a peak at 413 nm, indicative of a shift towards the low spin state.

Little electron transfer occurs in the absence of either CAM or antibody, but addition of CAM and $Ca^{+2}$ leads to the formation of a 450 nm band, indicative of flavin to heme electron flow. Addition of antibody in the absence of CAM (lower trace) leads to the formation of a 420 nm species. This suggests that antibody binding has activated electron transfer to the heme, but that it also places strain on the heme site conformation which results in the formation of the 'P420' type ferrous CO complex. Addition of dithionite results in the formation of the 450 nm species but does not significantly affect the concentration of the 420 nm CO complex.

The available evidence supports the conclusion that the insertion in the FMN binding domains of CNOSs is placed to interact with bound CAM, and that this insertion functions as a control element. The design of novel NOS inhibitors based on this premise is described below. Thus, ligands which activate electron transfer, and hence catalysis, can also be constructed.

Autoinhibitory Control Element in Calcium-dependent, Constitutive NOSs

As discussed above, the two constitutively expressed isoforms of NOS (CNOSs), first identified in neuronal cells (NNOS) and endothelial cells (ENOS), remain dormant until calcium/calmodulin ($Ca^{2+}$/CAM) binding is actuated by transient elevations in intracellular $Ca^{2+}$. This $Ca^{2+}$-dependent mode of regulation provides pulses of NO for moment-to-moment modulation of vascular tone and neurosignaling. In contrast, activity of the immunostimulant-induced isoforms of NOS (INOS) is $Ca^{2+}$-independent, providing continuous high-output NO generation for host-defense. A remarkably high affinity for CAM, even at basally low levels of intracellular calcium, is responsible for the $Ca^{2+}$-independence of INOS.

The major CNOS FMN module insertions are notably rich in charged residues and have an excess of positive charge. this is especially true of the ENOS isoform, which contains the motif RRKRK (SEQ ID NO: 10). Considerable homology exists between the CNOS insertions, particularly towards their N-termini. It is also apparent that some structural reorganization has taken place during evolution, which can allow the two or three positively charged residues (depending on species) in the NNOS equivalent of the RRKRK region to recognize a similar binding site. The pattern of conservation suggests that ENOS and NNOS insertions contain at least two motifs.

The CAM binding site is immediately adjacent to the N-terminal edge of the FMN binding domain. With CAM bound, the CAM recognition site can be in a helical conformation; steric constraints suggest that it extends almost directly away from the FMN binding domain. Models show the FMN binding domains of INOS and ENOS with CAM positioned above the N terminal strand of the FMN domain, relative to each other. There are 7-8 residues between the end of the CAM recognition site proper and the start of the initial strand of the $\beta$ sheet; 2-3 residues at each end of this short linker are needed to clear the van der Waals surfaces of CAM and the FMN domain. This leaves 2-3 residues which are conformationally unrestricted and, hence, there are uncertainties about the exact position above the $\beta$ sheet of CAM and the orientation of the axis of the CAM recognition site. The position of CAM relative to the FMN domain is unspecified with respect to rotations about the y axis by available information. It is notable that calmodulins ($M_r$ = 17 kDa) are larger than the entire FMN binding module. Although the insertion is midway through the sequence of the FMN-binding module, in three dimensions the model predicts it to be directly adjacent to the CAM binding site. The model predicts also that CAM binding

would be sterically hindered by the insertion, suggesting that the insertion can exist in more than one physiologically relevant conformation.

Two aspects of this model strongly suggest that the insert functions as a control element: (1) the correlation between $Ca^{+2}$/CAM control and the presence of the insertion, and, (2) the proximity of the CAM binding site to the insertion and the probable steric interactions which would ensue. The insert can act as an inhibitory polypeptide which is displaced by CAM binding. It differs from inhibitory polypeptides common to other CAM-dependent enzymes, and CAM itself, in its lack of acidic and hydrophobic amino acids; this makes direct binding of the insertion to CAM sites in NOS isoforms unlikely. Nonetheless, CAM could displace the polypeptide insert from a neighboring site by binding domain overlap or through allosteric effects.

Functional significance of the autoinhibitory insert of CNOSs was evaluated using a series of synthetic polypeptide fragments. Polypeptides corresponding to promising recognition sites such as the RRKRK (SEQ ID NO: 10) motif of the ENOS insert were synthesized in lengths ranging from six to thirty-three residues. Both ENOS- and NNOS-derived peptide fragments were evaluated; effects on NOS activity are summarized below. At concentrations of 50-100 $\mu$M, several polypeptide fragments of the CNOS insertions profoundly inhibited ENOS and NNOS activity. The most effective inhibitory polypeptides were from the ENOS insertion and contained the RRKRK (SEQ ID NO: 10) motif. HNNOS-derived polypeptides weakly inhibited ENOS, but were without effect on NNOS. While all peptides were less potent on INOS, significant inhibition was obtained with $ENOS_{601-633}$ (JX2, SEQ ID NO: 14) and $ENOS_{607-634}$ (JX3, SEQ ID NO: 15). Notably, inhibition of INOS activity by these peptides was rapid and complete within one minute of

addition. Because CAM is very tightly bound to INOS and has a remarkably slow off-rate, with little dissociation occurring even after boiling, inhibition probably occurs without CAM displacement.

Overlap of the CNOS polypeptide insert and CAM binding domains, suggested from molecular modeling, supports the conclusion that the insert obstructs CAM binding. Where this involves "docking" of the insert within CNOSs, synthetic homologs of the insert can similarly bind and interfere with CAM binding. Potent inhibition of [$^{125}$I]-CAM binding to NNOS was observed with insert-derived polypeptide fragments; relative peptide potency for inhibiting CAM binding mirrored that for blocking NNOS activation. $IC_{50}$ values for ENOS-derived peptide fragments ranged from 1-10$\mu$M, and potency increased as the RRKRK (SEQ ID NO: 10) motif was progressively lengthened to include up to 33 amino acids. Inhibition of NNOS activity and CAM binding by insert-derived peptides was fully reversed by excess CAM ($ENOS_{607-634}$, SEQ ID NO: 15), indicative of a competitive mode of inhibition. Thus, the greater apparent potency of peptides for inhibiting CAM binding vs. activity is explained by differences in assay conditions; lower CAM concentrations were used to assess binding (1 nM) vs. activity (100 nM). Inhibition of CAM binding by peptide could not be overcome by excess $Ca^{2+}$.

Conceivably, the synthetic peptides could interfere with CAM binding to NOS by interacting with either NOS or CAM itself. That NOS is the actual binding target for ENOS-derived insert peptides was demonstrated in two ways. First, direct binding of peptide to [$^{125}$I]-CAM, quantified in the absence of NOS, was undetectable at concentrations that inhibited >90% of CAM binding to NNOS. Second, ENOS-derived peptides markedly enhanced the dissociation rate of [$^{125}$I]-CAM from preformed complexes with NNOS. In this experimental setting, dissociated [$^{125}$I]-CAM is prevented

from reassociating with NOS by addition of a 3,000-fold molar excess of unlabelled CAM. Thus, in order for a synthetic ENOS-derived peptide to *eject* CAM from its binding site on NNOS, it must at least transiently form a ternary CAM-containing complex with NOS. This supports the conclusion that the binding domain of the putative ENOS autoinhibitory element on NNOS either overlaps or allosterically perturbs the CAM binding domain.

Previously described inhibitors with demonstrated selectivity for NOS influence the arginine site in a manner that can be detected as a loss in sites or binding affinity for the arginine analog, [$^3$H]-NNA. Thus, it is notable that the CNOS insert peptides inhibit NOS activity and CAM binding with a slight increase, rather than decrease, in [$^3$H]-NNA binding. Specificity of the insert peptides is also indicated by a lack of inhibition of either NOS activity or CAM binding with each of 5 synthetic peptides, 10-15 amino acids in length, derived from sites on the FMN binding domain of CNOSs which are distinct from the insert polypeptide.

Displacement of the insert peptide from an internalized binding site on CNOSs by CAM would conceivably enhance exposure of the insert to proteolysis. This hypothesis was tested by examining the pattern of peptide accumulation during limited trypsinolysis of both NNOS and ENOS, in the absence and presence of bound CAM.

Earlier, Sheta *et al.* showed that of 165 possible tryptic cleavage sites in rat NNOS, a single preferred cut site resides at $Arg_{727}$ within the CAM binding sequence. Cutting at this site has served as an effective means for isolation of distinct reductase and oxygenase domains. With CAM present but not bound (due to addition of the $Ca^{2+}$-chelator, EGTA) tryptic cleavage of NNOS occurs almost exclusively at a single site, consistent with $Arg_{727}$ within the CAM binding site. Accordingly, a time-dependent

accumulation of fragments with apparent $M_r$ 77kDa and 85 kDa, corresponding to C-terminal reductase and N-terminal oxygenase domains, respectively was found. When CAM was permitted to bind NNOS, by omission of EGTA, $Arg_{727}$ was protected from proteolysis and a novel tryptic cleavage site was revealed. Cutting at this new site yielded fragments of apparent $M_r$ 63kDa and 93kDa. Molecular mass refinement by matrix assisted laser desorption ionization spectrometry (MALDI-TOF) indicated the smaller fragment to be 64,809 ± 324 Da. This product is best explained by cleavage at $Arg_{855}/Lys_{856}$, a dibasic (RK) site within the insert peptide which predicts a C-terminal fragment of 65,071 Da. That this fragment originates from the C-terminus of NNOS is indicated by our finding that it is the predominant trypsinolysis product of the bacterial-expressed C-terminal reductase domain ($NNOS_{721-1429}$) but is not produced by trypsinolysis of the N-terminal oxygenase domain ($NNOS_{1-721}$). Confirmation of cleavage at $Lys_{856}$ is provided by direct sequence analysis of its ten N-terminal amino acids. In accord with our findings, a thorough analysis of NNOS trypsinolysis in the absence of bound CAM, indicates that $Lys_{856}$ becomes a cut site following initial cleavage within the CAM binding site at $R_{727}$. Since $Lys_{856}$ is protected from tryptic cleavage in the absence of CAM, but exposed when CAM is bound (or the CAM binding site is severed), CAM displaces the FMN domain insert peptide of NNOS.

A similar conclusion is drawn from study of ENOS fragmentation after limited trypsinolysis. With CAM absent, tryptic cleavage of ENOS yields four principal peptides of nominal $M_r$ 57, 60, 68 and 77 kDa. This pattern is rationalized by cleavage at $Arg_{518}$ within the CAM binding site ($ENOS_{1-518}$ = 56,877 Da, $ENOS_{519-1204}$ = 76,308 Da) and at a second site, likely to be $Lys_{545}$ which resides between the CAM binding site and insert peptide ($ENOS_{1-545}$

= 59,916; $ENOS_{546-1204}$ = 73,270. Exposure of $Lys_{545}$ and proximity to the CAM binding site is predicted in the above models, this site appears to be within a helix-turn transition at the edge of the beta sheet distant from the FMN binding site (an asterisk in the right, upper panel, denotes $Lys_{545}$). Lack of cleavage in NNOS at the site homologous to ENOS $Lys_{545}$ can be explained by the presence of a single basic residue, while ENOS contains paired basic residues (RK). In any event, binding of CAM simplifies this cleavage pattern by providing a single dominant cut site. Neglecting the intact proteins and the 10 kDa band from small unresolved fragments of ENOS, only two strong bands are visible at 60 and 65 kDa. These are predicted by cleavage of the molecule within the pentabasic RRKRK (SEQ ID NO: 10) motif in the insert peptide at residue $Lys_{632}$. Thus, cleavage at $Lys_{632}$ (with additional cleavage of the N-terminal fragment at $Lys_{545}$) produces fragments of 59,916 Da ($ENOS_{1-545}$) and 63,251 Da ($ENOS_{633-1204}$). Alignment reveals close correspondence between $Lys_{632}$ of ENOS and $Lys_{856}$ of NNOS, suggesting that CAM binding similarly displaces the insert peptide in each CNOS isoform.

To summarize, CAM binding not only protects the CAM binding site from degradation by trypsin, but exposes cleavage sites on both NNOS and ENOS which are otherwise inaccessible. A preponderance of evidence points to the clusters of basic residues in the FMN domain insert as the trypsin cleavage sites which are exposed by CAM binding. Exposure of cryptic sites by CAM binding could occur by an allosteric mechanism, or by displacement through binding domain overlap. CAM-driven movement of the insert strongly suggests a switch function for activation of NO synthesis.

Thus, CNOSs possess a polypeptide insert in their FMN binding modules that is: (1) unique to NOS isoforms which are regulated by transient CAM binding, (2) positioned adjacent to the CAM binding domain, (3) an impediment to

CAM binding and hence, NOS activation, and, (4) displaced with CAM binding does occur. Together, these results strongly support the conclusion that the insertion in CNOSs is an autoinhibitory control element. Inhibition of NOS by the insert requires occupancy of key sites on CNOS. CAM binding displaces the insert, thus activating CNOS catalysis by "disinhibition". Close proximity of the inhibitory polypeptide to its cognate binding site(s) on CNOSs would result in an exceedingly high local concentration, thus, favoring the bound/inhibited state in the absence of CAM. The detection of basal activity with either purified ENOS or NNOS, in the simultaneous presence of EGTA and absence of CAM can arise from a low steady-state concentration of the disinhibited CNOS conformer.

The control mechanism requires that CAM displace the insert upon binding to CNOS; this should translate into a reduced affinity for CAM. Reciprocally, absence of the insert from INOS would preclude the otherwise expected steric hindrance to CAM binding, contributing to the much tighter binding of CAM at low levels of $Ca^{2+}$. Studies of polypeptides, corresponding to the putative CAM binding sites on ENOS and INOS, and of chimeras in which the putative CAM binding sequence of one NOS isoform is substituted with the corresponding portion of another, have indicated that affinity and calcium-dependence of CAM binding is provided by elements on NOS in addition to the recognized CAM binding sequence itself. These results have been interpreted as indicating the presence of an auxiliary CAM binding region on INOS that augments binding. An alternative explanation, raised by the findings described herein, is that the absence of the autoinhibitory polypeptide from INOS contributes to enhanced CAM affinity at low $Ca^{2+}$ levels. Perhaps, INOS evolved from an ancestral CNOS-like protein by loss of the inhibitory peptide; nonetheless, vestigial regulatory sites are

suggested by a weak inhibition of activity in the presence of synthetic fragments of the ENOS inhibitory peptide. The CAM binding sites on INOS and CNOS are apparently related to a similar basic region near the N-terminus of CPNR, and may have evolved from such a region in a common ancestral protein.

Our data suggest that binding of the inhibitory peptide can involve at least two regions. At least one recognition site binds the RRKRK (SEQ ID NO: 10) motif. A second site can recognize sequences such as EERKSYKVRF (SEQ ID NO: 37) and EQHKSYKIRF (SEQ ID NO: 38) that occur in the N-terminal half of the ENOS and NNOS insertions; peptides that lack RRKRK (SEQ ID NO: 10) but contain these sequences weakly inhibit NOS activity and CAM binding. Some similarity between the first and second halves of the insertion can be readily noted by comparing the sequences of peptide $ENOS_{628-633}$ and $ENOS_{626-636}$ with those of $NNOS_{835-845}$ and $ENOS_{604-615}$. The insert peptide also contains an abundance of serine and threonine residues which provide potential sites for phosphorylation (12/45 residues in the bovine ENOS insert). Phosphorylation/dephosphorylation can also influence the affinity of insert peptides for binding cognate sites on CNOSs and hence, impact on parameters of NOS activation and/or deactivation. In this regard, it is notable that skeletal muscle possesses a NNOS splice variant in which the insert peptide is expanded by 36 residues, providing additional sites for possible cell-type specific modification.

Regions of interaction on the surface of the enzyme by the insert could include the flanking surface loops of the FMN-domain, bound CAM, and additional more distant sites. In particular, a site on the oxygenase domain consisting of an array of acidic groups could serve as a binding site for the basic regions on the inhibitory polypeptide insert which stabilizes the inhibited conformation of CNOS.

Confirmation of the regions of interaction can be exploited to manufacture additional inhibitory agents.

The C-terminal halves of the sequence of the FMN binding domains contain two sets of negatively charged residues. One such set, beginning with the sequence GQGDEL in ENOS, is conserved in all NOS sesquences and other enzymes in which both the FMN and FAD binding domains are present. The sequence VDTRLEELGGERL in ENOS is representative of a second set of acidic residues which are conserved in CNOSs but not in INOS or in P450 reductase, which typically contain only one of the four acidic groups. Part of this region was initially identified with the outer of the two flanking loops; advances in our understanding of the P450 reductase, and hence NOS, structure now make it clear that these residues extend along the edge of the FMN binding domain which interacts with the oxygenase domains, and that this region is close to both the inhibitory insert and the CAM binding site. This region is thus the best current candidate for a second regulatory element.

While it is clear that CAM binding and activation of CNOS is associated with displacement of the inhibitory polypeptide, it is not known how the presence of the polypeptide in its initial conformation inhibits electron transfer. One potential mechanism would involve interference by the inhibitory polypeptide with interactions between the oxygenase and reductase domains or flavin subdomains, stabilizing a conformation which does not support rapid electron transfer. Such interference could involve changes in either heme/FMN or FMN/FAD distances driven by domain realignment. Intramolecular electron transfer rates are often determined by the ability of electrons to tunnel, and therefore fall off exponentially with distance at roughly an order of magnitude per bond length; thus, a small increase in interdomain distance could produce a large reduction in

electron flux. Displacement of the inhibitory peptide may not be the only mechanism by which CAM binding stabilizes the activate conformation of CNOSs, inasmuch as CAM removal results in inactivation of INOS. This can be further confirmed and exploited in the manufacture of inhibitory agents.

In conclusion, a novel control element in CNOSs can serve in the design of potent peptide inhibitors. The exposure of tryptic cleavage sites in this element represents the demonstration of a specific CAM-induced conformational change in NOS and can be a hallmark of the active conformer of NOS. The use of CAM to control electron transfer is unique to CNOS. A more fundamental difference between CNOS and other CAM-regulated proteins is the lack of a CAM analog within the CNOS inhibitory peptide. Ultimately, CNOS may not be unique in this regard; it may presage the identification of other CAM regulated systems in which the CAM/inhibitor interaction is mediated through binding domain overlap or allosteric effects, rather than competition for a common recognition site.

General Screening Methods for Agents that Modulate NOS Activity

Based on the discovery of the regulatory regions of NOS isoforms, it is now possible to identify agents which modulate the activity of a nitric oxide synthase. An agent which "modulates" the activity of a nitric oxide synthase, as used herein, is an agent which either increases or decreases the activity of an NOS enzyme. Agents which increase the activity of an NOS enzyme are those which activate or promote the activity of the NOS enzyme. Agents which decrease the activity of an NOS enzyme are those which inactivate, interfere with, minimize or prevent the activity of the enzyme. Agents of the invention can

modulate NOS enzyme activity independently of calmodulin activation of the NOS enzyme; that is, whether or not calmodulin is associated with the NOS enzyme, it is the agent, rather than the calmodulin, that activates the NOS enzyme.

Current screening strategies for agents that modulate NOS activity are limited to methods that screen for agents that inhibit NOS enzyme activity. Screening methods for arginine (and pterin) analog inhibitors depend upon assays which are designed to define the effect on activity of the occupation of substrate (arginine) and/or cofactor (e.g., tetrahydrobiopterin). Conditions are selected to ensure that the limitation on enzyme activity is imposed at the catalytic site, and on arginine and N-OH arginine hydroxylation per se rather than on the preceding electron transfer reactions. A screen for an arginine analog NOS inhibitor includes arginine concentrations high enough to produce good activity for an extended time course in the absence of inhibition, but preferably not so high that strong inhibitors would be outcompeted by substrate for the binding site. All other conditions are adjusted to produce maximal or nearly maximal activity; the pterin binding site is saturated, and excess levels of calcium and calmodulin are included so that the arginine site occupancy fraction, and not electron transfer from NADPH, is rate limiting. The strategy for screening for a pterin based inhibitor is similar, except that the arginine concentration is preferably saturating, and the enzyme is preferably pretreated with pterin analogs to be screened because of the slow "off rate" of tetrahydrobiopterin. This can be accomplished with pterin replete enzyme by a long (e.g., one or more hours) preincubation with potential inhibitors, or with pterin free recombinant enzyme by a short (e.g., less than one hour) preincubation followed by reconstitution with tetrahydrobiopterin.

In contrast to these methods, screens for inhibitory agents that interact with the newly-identified regulatory regions of the NOS isoforms differ in that conditions are preferably chosen so that the rate limitation is imposed on the controlled steps, which are electron transfer reactions in which reduction of the catalytic heme group by NADPH is mediated by FMN and FAD. Conditions are selected so that the enzyme is replete with all cofactors and arginine, oxygen and NADPH are far above their Kd values for a sufficient time course to observe good kinetics. For inhibitor selection, NADPH consumption can be measured, as it is easier to measure than NO or citrulline production. If NADPH consumption is inhibited, NO and citrulline production must also be inhibited; however, the converse is not true, since under some conditions NADPH or oxygen electron transfer can be uncoupled from electron transfer.

The most important difference is the selection of calcium and calmodulin concentrations so that nearly maximal activity is obtained in the absence of the potential inhibitors, but also so that the control site is not supersaturated with $Ca^{2+}$-CAM. The excess levels of calcium and calmodulin (CAM) used in standard NOS assays to ensure complete activation are sufficient to mask the effects of fairly potent control site peptide inhibitors, because the binding domains of these inhibitors overlap that of $Ca^{2+}$-CAM, leading to competitive binding. The discovery of the regulatory regions, and the description of some of the properties of the regulatory regions and the related control sites of the NOS isoforms, as described herein, make it possible to select criteria to identify agents which interact with the newly identified active sites. Prior to the discovery of the regulatory regions of NOS enzymes, there was no theoretical basis to expect that such agents existed or could be isolated.

Bearing in mind these considerations, assays can be used to determine whether an agent modulates NOS activity. A sample of the agent to be tested (the "test agent") is contacted with a sample of NOS (thereby generating a test sample, herein referred to as a "synthase sample"); after incubation of the synthase sample under conditions appropriate for activity of the enzyme, as described above, the level of NOS activity is measured. The level of NOS enzyme activity can be, for example, compared to the amount of activity of a control sample of the NOS under the same conditions but in the absence of the test agent. The level of INOS activity, or of the constitutive NOS enzyme activity, is measured by any one of several methods; the choice of method depends on the intent of the assay, as described above. If the level of activity in the synthase test sample is different from the level of activity of a control sample of the NOS under the same conditions but in the absence of the test agent, then the agent modulates the activity of the NOS.

Similarly, assays can be used to determine whether an agent modulates the activity of one NOS isoform without modulating the activity of other NOS isoforms. For example, an agent can be assayed to test whether it modulates the activity of INOS, without modulating the activity of a constitutive NOS enzyme. A sample of the agent to be tested (the "test agent"), such as an antibody, peptide, or peptidomimetic described above, is contacted with a sample of INOS (thereby generating a test sample, herein referred to as am "inducible synthase sample"); after incubation of the inducible synthase sample under conditions appropriate for activity of the enzyme, the level of INOS activity is measured. The level of INOS enzyme activity can be, for example, compared to the amount of activity of a control sample, of INOS under the same conditions but in the absence of the test agent.

Similarly, a sample of the constitutive NOS enzyme is contacted with the test agent, to form a second test sample (herein referred to as the "constitutive synthase sample"); after incubation of the constitutive synthase sample under conditions appropriate for activity of the enzyme, the level of activity in the constitutive synthase sample is measured, and compared with the level of activity in a sample of the same constitutive NOS enzyme, under the same conditions, without the test agent. The level of INOS activity, or of the constitutive NOS enzyme activity, is measured by any one of several methods; the choice of method depends on the intent of the assay, as described above.

If the level of activity in the inducible synthase test sample is different from the level of activity of a control sample of INOS under the same conditions but in the absence of the test agent, and if the level of activity in the constitutive synthase test sample is approximately equal to the level of activity of a control sample of the constitutive NOS under the same conditions but in the absence of the test agent, then the agent modulates the activity of INOS, but does not modulate the activity of the constitutive NOS. Conversely, if the level of activity in the constitutive synthase test sample is different from the level of activity of a control sample of the constitutive NOS under the same conditions but in the absence of the test agent, and if the level of activity in the inducible synthase test sample is approximately equal to the level of activity of a control sample of INOS under the same conditions but in the absence of the test agent, then the agent modulates the activity of the constitutive NOS enzyme, and does not modulate the activity of INOS.

The selection of inhibitors with isoform specific properties involves comparison of inhibitory effects among assays involving all three isoforms. Such assays are

performed as described above, using a test and a control sample for each NOS enzyme. In selecting for NNOS or ENOS specific inhibitors, screens devised as described above are set up for NNOS and ENOS with the aim of selecting compounds which are significantly more potent inhibitors of one isoform than the other. An agent that "specifically inhibits" a particular NOS enzyme is an agent that inhibits that NOS isoform activity by approximately tenfold.

Screening Methods for Agents that Specifically Inhibit INOS

Because INOS lacks an analog of the autoinhibitory element of ENOS and NNOS, and is a much stronger binder of CAM; INOS binding of CAM is almost irreversible. Thus, during assays to identify agents that modulate INOS activity, excess CAM is not added: because in order to express active INOS, CAM must be coexpressed, and INOS is isolated replete with its CAM complement. INOS retains a site of interaction for inhibitory peptides, but because of the effectively zero off rate for CAM these peptides must bind to the INOS-CAM complex. Screens set up for assays of INOS which are analogous to the ENOS and NNOS assays mentioned above, except for the omission of added calcium and CAM, detect inhibitors which can form a ternary complex with INOS and CAM but not inhibitors which are strictly competitive with CAM. Strategies for an INOS screen which would detect inhibitors competitive with CAM include co-expression of a series of peptide inhibitors with INOS and CAM, or production of INOS in the presence of the inhibitor. This provides the opportunity for tight binding inhibitors to bind to the enzyme before CAM blocked their recognition site.

Additional Methods for Design and/or Identification of Agents that Inhibit Specific NOS Isoforms

NOS specific inhibitors can also be identified by using neighboring structural elements of the control site to design and screen for agents that interact with the control site. These elements in INOS include the short connecting loop that is specific to inducible nitric acid synthase (SEQ ID NO. 3), which occupies the position of the extended constitutive NOS insertion in the FMN domain, and the two flanking, negatively charged, loop sequences which correspond to the helix-strand transitions in INOS. An agent that binds to the loop that is specific to inducible NOS (SEQ ID NO. 3) occupies a position in INOS that is analogous to that of the regulatory element in constitutive NOS, and hence has potential inhibitory character. Agents that bind to the flanking loops can have binding domains which overlap the binding domain for the regulatory element, and can be inhibitory.

Similarly, alternative control site inhibitors for ENOS and NNOS have also been identified. Agents that bind to the corresponding flanking loop sequences in those isoforms can also serve as inhibitors. Such agents are now available. As described below, peptides corresponding to all the control site elements, both regulatory and structural, have been synthesized; antibodies to these peptides are examples of agents described above. Other agents can be identified by using a charge complement strategy; by screening an appropriate peptide library using an INOS inhibition assay; or by screening an appropriate peptide library using a selection strategy involving assaying for binding to the peptides.

Screening Methods for Agents that Activate ENOS and NNOS

Prior to the discovery of the regulatory region of the NOS isoforms, as described herein, it was not known that

NOS activation was possible by any means other than by CAM activation of the enzyme. Activation of constitutive NOS enzymes is particularly desirable, for treatment of diseases and conditions associated with NO production. Activation of ENOS and NNOS can most readily be visualized in terms of an agent that binds to the regulatory element of the isoform of interest, so as to prevent autoinhibition of NOS. An example of such an agent is an antibody to the regulatory element, which serves to interfere with the inhibited conformation. Other agents that bind to the regulatory element can be designed or identified by screening for activation. Design of agents utilizes charge complementarity: since a critical feature of the regulatory elements is the presence of an array of predominantly positive charges, activator candidates preferably have arrays of predominantly negative charges. Since extension of peptides containing positive charge arrays with additional residues corresponding to ENOS and NNOS regulatory elements which contain a few, or one negative charge, produce inhibitors of lower Kd than the positive charge array alone, flanking regions bearing one, or a few positive charges around a core of negative charges can be more potent as binding agents, and hence as activators.

The flanking loop sequences of all three NOS isoforms bear negative charges. In addition to their importance as sites for directed binding with regulatory potential, in constitutive NOS these sequences are part of the binding domain, and possibly part of the recognition site, of the regulatory peptide. In INOS, the corresponding sequences occupy the analogous positions spatially adjacent to the vestigial regulatory site. Structurally, the constitutive NOS flanking loops form the edges of a cup which surrounds the base of the regulatory peptide. This suggests that the negative charges on these loops may interact with some of

the positive charges on the regulatory peptide, and hence the synthetic peptides derived from these loops are examples of the agents bearing negative charge arrays discussed above. Thus, they can be CAM independent activators.

Screens for CAM independent control site activation of a NOS enzyme uses assays in which conditions are selected for nearly maximum activity, except that no CAM is added. This includes saturation of the enzyme with arginine, oxygen and NADPH, and, if necessary, reconstitution with tetrahydrobiopterin. Potential activators are selected for their ability to stimulate NO production. While it is possible to use NADPH consumption as an initial assay for this screen, it is preferable to use NO (or citrulline) formation as a basis for the assay, as an increase in NADPH consumption could be due to an increase in the uncoupled rate of electron transfer to oxygen.

Design of Agents That Bind to Control Sites of NOS Isoforms

Based on the discovery of the regulatory region of the NOS isoforms, agents that bind to the NOS isoforms control sites can be developed. Initial agents that were designed and tested, as described below in the Examples, were synthetic variants of the autoinhibitory elements of constitutive NOS isoforms, neighboring structural elements, and complements (including antibodies) to these elements. The agents include peptides which have been identified as potent inhibitors, and agents having the same or substantially the same characteristic properties of these inhibitors, including charge arrays such as the polybasic sequences found in the control elements and their complements.

Developmental strategies for agent design take several forms. Starting with the existing inhibitory peptides, homologous or similar peptides are sought which increase

the potency of inhibition, or which improve isoform specificity. These homologous or similar sequences can be identified either by mass screening of related sequences or by sequential variations to rationally design a superior variant. Furthermore, peptidomimetics can be devised utilizing the established importance of the polybasic array in inhibitor potency. For example, a peptidomimetic can be designed by substituting the peptidic backbone of the molecule with an alternative organic or inorganic backbone, whereby the basic moieties are arranged and/or presented in the same three-dimensional configuration. Steric hindrance can be introduced to improve specificity and libraries of charge complemented (primarily negative) molecules can be screened to find agents that bind to the control site elements themselves.

Agents of the Invention

Agents which modulate NOS enzyme activity include agents having an array of positively charged residues or molecules, such as the array of positively charged amino acids in ENOS-homologous peptides. This agent can modulate a NOS enzyme activity by effecting electron transport between NADPH and the active site of an NOS enzyme. Other agents have at least one functional group, and may have two or more functional groups, which bind to the INOS-specific peptide; or have at least one functional group, and may have two or more functional groups, which bind to the recognition site of the inhibitory peptide in a constitutive NOS enzyme, or a homologous region in inducible NOS, and thereby modulate INOS activity. The functional group(s) of the agent can be, for example, amine groups, which bind to a carboxyl group within the INOS-specific peptide. For example, as described in Example 2 below, certain peptides of the invention have been shown to

inhibit the activity of INOS. These peptides are useful as agents which modulate INOS activity.

In addition, agents that bind to neighboring structural elements can also serve as either activators or inhibitors by interfering with CAM binding, with the active or inactive conformation of the intrinsic autoinihibitory element, or by sterically mimicking the inhibitory effect of the autoinhibitory element. For example, the negatively charged flanking loop regions form the NOS FMN binding domains are neighboring structural elements that can serve as activators or inhibitors of NOS isoforms. Representative agents include antibodies for the synthetic peptide analogs of the NOS control elements and neighboring structural elements. For example, antibodies which are raised to the INOS-specific polypeptide (SEQ. ID. NO. 3) and/or related regions, can be used as agents which modulate INOS activity without modulating the activity of a constitutive NOS enzyme, because they specifically bind to INOS, but not to a constitutive NOS enzyme. Antibodies raised to polypeptides comprising one or both of the two negatively charged loops on the surface of the FMN binding molecule present in all known NOS isoforms, also modulate INOS activity without modulating the activity of a constitutive NOS enzyme. The two negatively charged loops are exposed in INOS, but are not in the constitutive NOS enzymes, because the sites appear to serve as the binding site for the regulatory peptides in ENOS and NNOS, and are therefore covered by the positively charged regulatory peptide, or by the regulatory peptide and calmodulin. Antibodies raised to a NOS regulatory peptide (such as SEQ. ID. NO. 1 or NO. 2), or a polypeptide comprising the NOS regulatory peptide and/or related regions, can be used as agents which modulate the activity of constitutive NOS enzymes without modulating the activity of INOS, as they

specifically bind to constitutive NOS enzymes, but not to INOS.

In preferred embodiments, the agent modulates the activity of INOS, without modulating the activity of a constitutive NOS enzyme (ENOS and/or NNOS); alternatively, the agent modulates the activity of constitutive NOS enzymes, without modulating the activity of INOS. In an even more preferred embodiment, the agent modulates the activity of one constitutive NOS enzyme (ENOS or NNOS), and does not modulate the activity of the other constitutive NOS enzyme. The agents of the invention include peptides, as well as antibodies, and peptidomimetics which have the same or similar activity as the peptides, as described below.

Peptides

A series of peptides has been constructed and tested for the ability to modulate the activity of (e.g., inhibit and/or activate) the various NOS isoforms. Thus, these substantially isolated peptides relating to the regulatory peptides of NOS proteins, as well as other related peptides can serve as agents that modulate NOS activity. A "substantially isolated" peptide, as described herein, refers to a peptide that has been derived or removed from the environment in which it naturally occurs. Examples include a peptide fragment derived or removed from the native protein, comprising or consisting essentially of the cited sequences. For example, a substantially isolated peptide would include the cited sequence, the sequence flanked by one or more amino acids which are the same as or different from the amino acid sequence of the native protein. A peptide that inhibits a NOS enzyme is referred to herein as a "nitric oxide synthase inhibitor peptide;" a peptide that activates a NOS enzyme is referred to herein as a "nitric oxide synthase activator peptide".

The peptides of the invention include the ENOS regulatory peptide, MSGPYNSSPRPEQHKSYKIRFNSVSCSDPLVSSWRRKR KESSNTD (SEQ ID NO. 1); the NNOS regulatory peptide, MRHPNSVQEERKSYKVRFNSVSSYSDSRKSSGDGPDLLRDNFE (SEQ ID NO. 2); the INOS specific peptide (SEQ ID NO. 3); the regulatory region between about amino acids 590-650 of ENOS; the regulatory region between about amino acids 820-880 of NNOS; and the negatively charged loops of INOS (amino acids 568-581 (SEQ ID NO. 4) and 633-647 (SEQ ID NO. 5)); ENOS (amino acids 557-570 (SEQ ID NO. 6) and 666-680 (SEQ ID NO. 7); and NNOS (amino acids 790-803 (SEQ ID NO. 8) and 897-911 (SEQ ID NO. 9). The peptides of the invention additionally include derivatives of the ENOS regulatory peptide, NNOS regulatory peptide, INOS specific peptide, regulatory regions of ENOS and NNOS, and negatively charged loops of INOS, ENOS and NNOS. A "derivative" of a peptide, as described herein, is a peptide which has one or more amino acids deleted or inserted, or has one or more conservative substitutions. A preferred derivative has one, two or more flanking amino acids (e.g. met or native NOS amino acids). For example MEMSGPYNSSPRPEQHKSYKIRFNSV SCSDPLVSSWRRKRKESSNTD (SEQ ID NO: --) A "conservative substitution", as used herein, is the replacement of a first amino acid with a second amino acid that is similar to the first amino acid in charge, polarity, reactivity, and/or structure. Conservative substitutions include amino acid substitutions within the following groupings: S, T, G, A, and P; L, M, I, and V; E, D, Q, and N; R, H, and K; and F, Y, and W. Fragments of these peptides can also serve as agents that modulate NOS activity. A fragment of a peptide that inhibits NOS activity is referred to herein as an "inhibitory fragment;" a fragment of a peptide that activates NOS activity is referred to herein as an "activating fragment."

The invention further encompasses peptides that are closely related to the ENOS control region, NNOS control region, INOS specific peptide, regulatory regions of ENOS and NNOS, and negatively charged loops of INOS, ENOS and NNOS, as shown by the degree of homology, and, in the case of some peptides based on autoinhibitory elements, by the presence of a core region containing an array of positively charged amino acids. These closely related peptides are referred to herein as "homologous peptides". The core region of a peptide homologous to the ENOS regulatory peptide contains an array of positively charged amino acids. The array has at least two, and preferably at least five, positively charged amino acids. At least two of the positively charged amino acids must be contiguous. The positively charged amino acids can be naturally-occurring or synthetic. In a preferred embodiment, each positively charged amino acid is lysine, arginine, histidine, or ornithine. In a more preferred embodiment, the core region is RRKRK (SEQ ID NO. 10); in another preferred embodiment, the core region is KKRKR (SEQ ID NO. 11). The homologous peptides of the invention are at least 60% homologous, or about 67% homologous, or about 80% homologous, or about 90% homologous to either a section of the ENOS regulatory peptide (SEQ ID NO. 1), the NNOS regulatory peptide (SEQ ID NO. 2), or the INOS specific peptide (SEQ ID NO. 3). The percent of homology indicates the amount of identity (or similarity) between the amino acid sequences of two peptides. The percent similarity takes into consideration close or conservative substitutions in amino acids. In a particularly preferred embodiment, the sequence of the homologous peptide is substantially the same as the identified sequence.

In one embodiment, a homologous peptide of the invention is a substantially isolated peptide having an array of positively charged amino acids, and an amino acid

sequence of at least about 60% homology, or about 67% homology, or about 80% homology, or about 90% homology, or is substantially the same as, the amino acid sequence of the ENOS control region (SEQ ID NO. 1). In a preferred embodiment, the substantially isolated peptide binds to a site spatially adjacent to the calmodulin binding site of ENOS. Particular peptides include WRRKRK (SEQ ID NO. 12); SSWRRKRKESS (SEQ ID NO. 13); SSPRPEQHKSYKIRFNSVSCSDPLVSSWR RKRK (SEQ ID NO. 14); and QHKSYKIRFNSVSCSDPLVSSWRRKRKE (SEQ ID NO. 15). Also SSPRPEQHKSYKIRFNSVSCSDPLVSSWRRKRKESS (SEQ ID NO: 38). An additional preferred peptide includes the entire autoinhibitory peptide insert.

In another embodiment, a homologous peptide of the invention is a substantially isolated peptide having an amino acid sequence of at least about 60% homology, or about 67% homology, or about 80% homology, or about 90% homology to, or substantially the same as, the amino acid sequence of the NNOS control region (SEQ ID NO. 2). In a preferred embodiment, the substantially isolated peptide binds to a site that is spatially adjacent to the calmodulin binding site of NNOS. Particular peptides include QEERKSYKVRF (SEQ ID NO. 16), RPEQHKSYKIRF (SEQ ID NO. 17), SDSRKSSGDGPDLR (SEQ ID NO. 18), and QEERKSYKVRFNSVSSYSDSQKSSGDGPDL (SEQ ID NO. 19).

In further embodiments, other homologous peptides include a substantially isolated peptide having an amino acid sequence of at least about 60% homology, or about 67% homology, or about 80% homology, or about 90% homology to, or substantially the same as, the amino acid sequence of the INOS specific peptide (SEQ ID NO. 3); and a substantially isolated peptide having an amino acid sequence of at least about 60% homology, or about 67% homology, or about 80% homology, or about 90% homology to the negatively charged loops of the NOS enzymes (SEQ ID NO. 4 and 5 (INOS); 6 and 7 (ENOS), and 8 and 9 (NNOS)).

The peptides of the invention can be prepared using conventional synthetic methods. Alternatively, they can be prepared using conventional methods of molecular genetics (see Sambrook, J. *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY (1989)). The peptides of the invention can also be prepared as fusion peptides with other peptide or proteins, or with other macromolecules, such as polysaccharides.

Antibodies to the Peptides

The peptides of the invention can be used to raise antibodies. Antibodies which are raised to the peptides can be either monoclonal or polyclonal. The term "antibody", as used herein, encompasses both polyclonal (e.g. murine or rabbit) and monoclonal antibodies, as well as mixtures of more than one antibody (e.g., a cocktail of different types of monoclonal antibodies) reactive with a peptide of the invention. The term antibody is further intended to encompass whole antibodies and/or biologically functional fragments thereof. Biologically functional antibody fragments which can be used are those fragments sufficient for binding of the antibody fragment to the peptide. Once the antibodies are raised, they are assessed for the ability to bind to the peptide. Conventional methods can be used to perform this assessment.

Monoclonal antibodies (mAb) reactive with a peptide of the invention can be produced using somatic cell hybridization techniques (Kohler and Milstein, *Nature 256*: 495-497 (1975)) or other techniques. In a typical hybridization procedure, a crude or purified NOS enzyme or a peptide of the invention as described above, (e.g., INOS-specific polypeptide, ENOS regulatory peptide, NNOS regulatory peptide, a homologous peptide, or a derivative peptide, as described above), can be used as the immunogen. An animal is immunized with the immunogen to obtain antibody-

producing spleen cells. The species of animal immunized will vary depending on the specificity of mAb desired. The antibody producing cell is fused with an immortalizing cell (e.g., a myeloma cell) to create a hybridoma capable of secreting antibodies to the peptide of the invention. The unfused residual antibody-producing cells and immortalizing cells are eliminated. Hybridomas producing desired antibodies are selected using conventional techniques and the selected hybridomas are cloned and cultured.

Polyclonal antibodies can be prepared by immunizing an animal in a similar fashion as described above for the production of monoclonal antibodies. The animal is maintained under conditions whereby antibodies reactive with the peptide of the invention are produced. Blood is collected from the animal upon reaching a desired titer of antibodies. The serum containing the polyclonal antibodies (antisera) is separated from the other blood components. The polyclonal antibody-containing serum can optionally be further separated into fractions of particular types of antibodies (e.g., IgG, IgM).

Antibodies raised to either the regulatory peptide of a constitutive NOS enzyme (ENOS or NNOS), or to the INOS-specific polypeptide, can be used to distinguish constitutive NOS enzymes from INOS, and vice versa. NNOS can be distinguished from ENOS and INOS, because NNOS has a long N terminal extension that is lacking in ENOS and INOS; antibodies to the regulatory peptide or to the INOS-specific polypeptide can now be used to distinguish ENOS from INOS. The potential for cross reactivity between NOS isoforms exists for antibodies to all other regions on the NOS surface except the regulatory peptide and the INOS-specific polypeptide. Antibodies can also be used as agents which alter the activity of a NOS protein, as described further below.

Peptidomimetics and Other Agents

Peptidomimetics (molecules which are not polypeptides, but which mimic aspects of their structures to bind to the same site), that are based upon the above-described peptides, can also be generated. For example, polysaccharides can be prepared that have the same functional groups as the peptides of the invention, and which interact with NOS enzymes in the same manner. Peptidomimetics can be designed, for example, by establishing the three dimensional structure of the peptide agent in the environment in which it is bound or will bind to the enzyme. The peptidomimetic comprises at least two components, the binding moiety or moieties and the backbone or supporting structure.

The binding moieties are the chemical atoms or groups which will react or complex (as in forming a hydrogen or covalent bond) with the amino acid(s) at the binding site of the enzyme. In general, the binding moieties in a peptidomimetic are the same as the peptide agent. Alternatively, the binding moieties can be an atom or chemical group which react with the enzyme in the same or similar manner. Examples of binding moieties suitable for use in designing a peptidomimetic for a basic amino acid in a peptide are nitrogen containing groups, such as amines, ammoniums, guanidines and amides or phosphoniums. Examples of binding moieties suitable for use in designing a peptidomimetic for an acidic amino acid can be, for example, carboxyl, lower alkyl carboxylic acid ester, sulfonic acid, a lower alkyl sulfonic acid ester or a phosphorous acid or ester thereof.

The supporting structure is the chemical entity that, when bound to the binding moiety or moieties, provides the three dimensional configuration of the peptidomimetic. The supporting structure can be organic or inorganic. Examples of organic supporting structures include polysaccharides,

polymers or oligomers of organic synthetic polymers (such as, polyvinyl alcohol or polylactide). It is preferred that the supporting structure possess substantially the same size and dimensions as the peptide backbone or supporting structure. This can be determined by calculating or measuring the size of the atoms and bonds of the peptide and peptidomimetic. In one embodiment, the nitrogen of the peptide bond can be substituted with oxygen or sulfur, thereby forming a polyester backbone. In another embodiment, the carbonyl can be substituted with a sulfonyl group or sulfinyl group, thereby forming a polyamide. Reverse amides of the peptide can be made (e.g., substituting one or more -CONH- groups for a -NHCO- group). In yet another embodiment, the peptide backbone can be substituted with a polysilane backbone.

These compounds can be manufactured by known methods. For example, a polyester corresponding to the peptide RRKRK (SEQ ID NO: 10) can be prepared by the substituting a hydroxyl group for each corresponding amine group on the R and K amino acids, thereby preparing a hydroxyacid and sequentially esterifying the hydroxyacids, optionally blocking the basic side chains and acids to minimize side reactions. Determining an appropriate chemical synthesis route can generally be readily identified upon determining the chemical structure using no more than routine skill.

In addition, large libraries of agents, such as those libraries that can be constructed using well-known methods of combinatorial chemistry, can be assayed for additional agents. Such agents can be isolated through methods described herein and are considered to be equivalent to the described agents, in that they interact with the same site on the NOS protein, fulfill the same function (e.g., alter the activity of (inhibit or activate) a specific isoform), and therefore, can be used to treat one or more diseases in which the inhibition or activation of a specific NOS

isoform is beneficial. This disclosure therefore encompasses such agents as can be prepared by one skilled in the art through the use of the discoveries and methods described herein.

Probes of the Invention

Isolated nucleic acid probes, which optionally may encode the peptides of the invention, as described above, can also be prepared. Nucleic acid probes, particularly those encoding or corresponding to the regulatory peptide of the constitutive NOS enzymes, ENOS and NNOS (SEQ. ID. NO. 1 and NO. 2) or to the INOS-specific polypeptide (SEQ. ID. NO. 3), can be used as markers for identifying and mapping NOS enzyme isoforms in humans and other organisms, as well as for probes for specific NOS enzyme genes and mRNA, and for identification of other enzymes with undiscovered but related regulatory sites. The nucleic acid probes can comprise RNA, cDNA, or genomic DNA. The nucleic acid probes substantially correspond to the native nucleic acids encoding or substantially corresponding to the regulatory peptide of the constitutive NOS enzymes, ENOS and NNOS (SEQ. ID. NO. 1 and NO. 2), the INOS-specific polypeptide (SEQ. ID. NO. 3), or derivative or homologous peptides.

To identify NOS enzyme isoforms or related enzymes, a DNA or RNA sample (a test sample) is obtained from the organism of interest; the test sample is contacted with a nucleic acid probe, under hybridization conditions which allow hybridization of the nucleic acid probe to homologous DNA or RNA in the test sample. Stringency hybridization conditions can be adjusted to eliminate hybridization to extraneous sequences (see Sambrook, J. *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY (1989)). The presence (or absence) of hybridization is then detected, using conventional

methods. The homologous DNA or RNA, if present, can be isolated for further study.

Nucleic Acid Molecules of the Invention

Isolated nucleic acid molecules which encode the peptides of the invention, as described above, as well as nucleic acid molecules complementary to the nucleic acid molecules, can also be prepared, using standard techniques (see Sambrook, J. *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY (1989)). Such isolated nucleic acid molecules can be used to prepare peptides of the invention. In addition, isolated nucleic acid molecules which encode the antibodies of the invention, as described above, as well as complementary nucleic acid molecules, can also be prepared and used to generate antibodies of the invention.

Methods of Treatment

The agents that modulate NOS enzyme activity, as described above, can be used to modulate the activity of a nitric oxide synthase enzyme *in vivo*. In a preferred embodiment, the agent is used to modulate the activity of a nitric oxide synthase in a mammal, such as a human, in order to treat a disease or condition associated with NO production in the mammal. An agent which modulates the activity of INOS, or of a constitutive NOS enzyme (NNOS or ENOS), is administered to the mammal. The agent can be administered subcutaneously, intravenously, intramuscularly, intraperitoneally, topically, orally, rectally, nasally, buccally, vaginally, intraurethrally, by inhalation spray, or via an implanted reservoir. Where the agent is a peptide, the agent can also be administered via a gene transfer vector containing a nucleic acid encoding the peptide. Administration of the gene transfer vector leads to expression of a nucleic acid sequence, resulting

in production of the peptide and modulation of the specific NOS isoform. A gene transfer vector containing a nucleic acid encoding the peptide can also contain tissue-specific promoters, as well as other elements (e.g., enhancer elements, splicing signals, termination and polyadenylation signals, viral replicons, bacterial plasmid sequences, or other vector nucleic acid sequences). Delivery of the vector can be targeted to particular regions or cell types (e.g., by the use of decorated liposomes, or by introducing the vector in a specific region). For example, transformation of cells with a peptide that inhibits INOS activity would be effective protection against NO-related islet cell destruction in those at risk for early onset diabetes. In another example, transformation of endothelial cells with a peptide that activates ENOS can be used for long-term treatment of hypertension.

If the agent is an antibody, gene transfer methods can also be used to administer a nucleic acid encoding the antibody or a fragment of the antibody. Administration of the vector leads to expression of the nucleic acid sequence, resulting in production of the antibody, and, ultimately, modulation of the specific NOS isoform.

The agent can be administered in dosage formulations containing conventional, non-toxic, physiologically-acceptable carriers, adjuvants, and/or vehicles. The form in which the agents are administered will depend at least in part on the route by which they are administered.

The agent is administered in an effective amount, which is that amount necessary to modulate the NOS enzyme. In treatment of a disease or condition, the agent is administered in a therapeutically effective amount. A therapeutically effective amount is that amount necessary to reduce or eliminate symptoms associated with the disease or condition. The effective amount, or the therapeutically effective amount, will be determined on an individual

basis, and will be based in part, on consideration of the agent, the individual's size and gender, the severity of the symptoms to be treated, the result sought, the disease, etc. Thus, the therapeutically effective amount can be determined by one of ordinary skill in the art, employing such factors and using no more than routine experimentation.

The therapeutically effective amount can be administered in a single dose, or a series of doses separated by appropriate intervals, such as hours, days, or weeks. The term "single dose," as used herein, can be a solitary dose, and can also be a sustained release dose, such as by a controlled-release dosage formulation or a continuous infusion. Other drugs can also be administered in conjunction with the agent; e.g., a vasopressor administered in conjunction with an INOS inhibitor for septic shock treatment. More than one agent which modulates the activity of a NOS enzyme can be administered at the same time.

In one embodiment of the invention, an agent which modulates, and particularly, which increases the activity of ENOS, is administered, in order to treat a condition modulated by production of nitric oxide by endothelial nitric oxide synthase, such as hypertension, atherosclerosis, or acute asthma. In a preferred embodiment, the agent does not modulate the activity of NNOS or INOS. An agent which increases ENOS activity will allow NO to be produced directly adjacent to the site of action. Since such an activator would not have to dissociate to produce NO, it would be stable and would not need to be "used up" to be effective. This is due to the mode of action of the agent; it turns on a catalyst, and each molecule of agent can cause the production of many molecules of NO gradually over a long time course. In contrast, NO donors such as nitroglycerin are limited by

the number of NO groups they contain (usually 1-3) and must be replaced as they are used up to produce NO.

In the case of constitutive NOS enzymes (NNOS or ENOS), an agent which activates either ENOS and/or NNOS in the corpus cavernosa can be used as a means for treating male erectile dysfunction. In one embodiment, the agent is administered intraurethrally to limit systemic side effects.

In another embodiment, an agent which specifically inhibits NNOS can be used to prevent brain damage in conditions involving cerebral ischemia/reperfusion injury, such as head trauma.

In another embodiment, an agent which modulates, and particularly, which decreases the activity of INOS is administered in order to treat a condition modulated by production of nitric oxide by INOS, such as septic shock, toxic shock, autoimmune disease such as rheumatoid arthritis, inflammatory conditions such as inflammatory bowel disease, multiple sclerosis, or diabetes. In a preferred embodiment, the agent which modulates INOS activity does not modulate the activity of NNOS or ENOS. Furthermore, INOS is important in dementia associated with Acquired Immune Deficiency Syndrome (AIDS), and is involved in the destruction of the immune system and general physical deterioration produced by the AIDS virus (Baldeway, T., *et al.*, *AIDS 10*451-452 (1996); Brenner, T., *et al.*, *Brain Res. 641*:51-56 (1994); Lipson, S.A., *Devel. Neurosci. 94*:145-151 (1996); Buck, M. *et al.*, *EMBO J. 15*1753-1763 (1996); Cross, *et al.*, *J. Clin. Invest. 93*:2684-2650 (1994); Boulleme, A.I. *et al.*, *J. Neuroimmunol. 60*:117-124 (1995); Mayer, M., *Nervenarzt 65*:819-827 (1994)). Therefore, an agent which modulates the activity of INOS can be administered to combat dementia, immune system destruction, and/or physical deterioration in individuals infected with the AIDS virus.

Further, an agent which activates INOS can be used to treat diseases relating to dysplasia, cancer, or infectious disease. Activation of INOS can produce cytotoxic levels of NO which would aid in the elimination of dysplastic or cancerous tissue, or aid in the control of infectious agents such as viruses, microbes, or other parasites.

The following examples are further illustrative of the present invention.

EXAMPLE 1 Identification of the Nitric Oxide Synthase Regulatory Region

A. Structure of Nitric Oxide Synthase Enzymes

Discussed in more detail above, all nitric oxide synthase (NOS) enzymes are modular in nature: they consist of a series of connected regions, each of which is closely related to a small simple protein. The C-terminal region of NOS is homologous to two flavoproteins, ferredoxin NADP reductase (a flavin adenine dinucleotide (FAD)-linked enzyme), and flavodoxin (a small flavin mononucleotide (FMN) binding protein). The corresponding regions in NOS function together to bring electrons into the enzyme for production of NO from arginine and oxygen. Binding of calmodulin by NNOS and ENOS turns on electron transfer from the flavin binding region to the active site, which contains a heme cofactor. The calmodulin binding site in all three types of NOS is located close to the N-terminal edge of the flavodoxin module.

Almost immediately (within ten residues) after the calmodulin binding site in each NOS there is a region with the sequence TETGKSEALA (SEQ ID NO. 20) or a close homologue. This sequence is recognized as the homologue of closely related flavodoxin sequences such as TDTGKTEALA (SEQ ID NO. 21), which are involved in binding the flavin cofactor. These sequences form a dense hydrogen bond

network with the phosphate group of flavin mononucleotide (FMN).

B. Alignment of Sequences

The sequences of about twenty flavodoxins were aligned with the corresponding regions of all available NOS sequences and with the sequences of NADPH cytochrome P450 reductase (NCPR). NCPR is a related flavoprotein which, like NOS, has both the FMN and FAD binding domains, but is unregulated and lacks a calmodulin binding site. All known NOS isoforms contain two negatively charged loops on the surface of the FMN binding molecule. INOS itself contains a surface polypeptide not found in either ENOS or NNOS. This surface polypeptide, having the sequence of amino acids 600-615 of INOS (SEQ ID NO. 3), is referred to herein as the "INOS-specific polypeptide".

Computer alignment of the flavoprotein sequences failed to align or identify the TETGKSEALA (SEQ ID NO. 20) regions because of a large insertion in the constitutive NOS enzymes (NNOS and ENOS), and because available programs do not make use of three dimensional structural information. The TETGKSEALA (SEQ ID NO. 20) region and the previously recognized conserved sequences which make up the FMN binding site were aligned by hand. The Figure shows an alignment of a selected set of NOS, NCPR and bacterial flavodoxin sequences. NCPR HUMAN, human NADPH P450 reductase (SEQ ID NO. 29); NOSE BOVIN, bovine ENOS (SEQ ID NO. 30); NOSB RAT, rat NNOS (SEQ ID NO. 31); NOSM MOUSE, mouse INOS (SEQ ID NO. 32); FLAV ECOLI, *Escherichia coli* flavodoxin (SEQ ID NO. 33); and FLAV DESVH, *Desulfovibrio vulgaris* flavodoxin (SEQ ID NO. 34). Critical regions, which were identified based on the three dimensional structures of flavodoxins that have been solved by X-ray crystallography were forced to align.

C. Identification of Regulatory Region

The pattern of the insertions, identified by the alignment, revealed the control mechanism of nitric oxide synthase. Compared to flavodoxins, which lack a calmodulin binding side, NCPR has no insertions of more than five residues after the TETGKSEALA (SEQ ID NO. 20) region in the FMN binding domain. Immune (macrophage) NOS (INOS or MNOS), which binds calmodulin but does not respond to calcium, also lacks an insertion in the corresponding region. In contrast, the constitutive NOS isoforms (ENOS and NNOS), which bind calmodulin and are under calcium control, have extensive (40-45 residues) insertions within the FMN binding domain. This is the critical difference between the constitutive and inducible isoforms of NOS.

The insertions are about 100 residues downstream (towards the C terminus) from the calmodulin binding site. On the published three-dimensional structure of flavodoxin (Wapenpaugh, K., *et al.*, *PNAS* 70:3852-3860 (1973)), this insertion lies on an external loop at one edge of the beta sheet which forms the core of the protein. The insert is spatially adjacent to the calmodulin binding site.

Since the insert is about 30% as large as a flavodoxin molecule, and calmodulin is comparable in size to flavodoxin, it is clear that steric effects are important. Calmodulin binding to INOS is unusually tight, partly because of the absence of interference from the insert. In ENOS and NNOS, since the insert and calmodulin cannot occupy the same space, calmodulin binding forces the insert out of the way. It is this interaction between calmodulin and the insert which turns the enzyme on. Thus, ligands which interact with the insert and the adjacent region of the FMN domain will therefore tend to force the switching mechanism into the off state or the on state. In other calmodulin regulated systems, such ligands may produce a

weakly active state in the absence of calmodulin; this weakly active state is resistant to further activation. The negatively charged loops on the surface of the FMN binding domain appear to serve as the binding site for the regulatory peptides in ENOS and NNOS. The regulatory polypeptide in INOS is the short loop which is split and greatly extended by the introduction of the regulatory peptides in ENOS and NNOS.

D. Structural Models

The availability of solved X-ray structures for flavodoxins allows us to position the insertion in three dimensions relative to the calmodulin binding site. Homology-based molecular models were constructed for the FMN binding domains of constitutive NOS, INOS and p450 reductase. These models were constructed using the Insight and Homology programs (Biosym, Inc.), and could be relaxed to a sterically and energetically reasonable state using Discover software (Biosym, Inc.).

The backbones of the INOS and p450 reductase modules can be almost superimposed on the backbone of Desulfovibrio flavodoxin, which appears to be the closest solved structural homolog of the FMN binding modules of the NOS isoforms. The structure, a Rossman fold motif, is a five-stranded, parallel $\beta$ sheet, with the FMN binding site along one edge. Two aromatic residues, W and Y in INOS, are in contact with the FMN ring system; the latter serves as a shielding residue.

Most of the corresponding backbone structure of ENOS can be superimposed on the structures of INOS and p450 reductase modules, but the insertion projects from the edge of the sheet opposite the FMN binding site. The backbone of NNOS is similar to ENOS. Structurally, the insertion corresponds to the replacement of a tight 5-10 residue $\alpha>\beta$ loop with an approximately 50 residue structure which is

about 1/3 the size of the entire FMN binding module. The conformation for the insertion is currently unknown.

The calmodulin binding site is directly adjacent to the N terminal edge of the FMN binding domain. When calmodulin is bound, the site must be in a helical conformation, and can then be expected to extend almost directly away from the FMN binding domain because of steric constraints. Calmodulins (m.w. approximately 20 kD) are considerably larger than the entire FMN binding module. Although the insertion is midway through the sequence of the FMN binding module, it is immediately apparent that in three dimensions it is directly adjacent to the CAM binding site. Clearly, CAM binding is likely to be sterically hindered by the insertion, and probably demands that the insertion undergo conformational reorganization. It is therefore likely that the insertion has more than one physiologically relevant conformation.

The proximity of the CAM binding site to the insertion, their probable steric interactions, and the correlation between calcium/calmodulin control and the presence of the insertion strongly suggest that the insertion functions as a control element. The role is that of an inhibitory polypeptide displaced by CAM binding. It would differ from the inhibitory polypeptides in other systems, in that it is not a CAM analog, but would be displaced from a neighboring site because of binding domain overlap.

EXAMPLE 2 Synthetic Polypeptide Effects

In order to evaluate the functional significance of the polypeptide, a series of synthetic polypeptides were designed which incorporated structural features of loop regions in the FMN domain. Polypeptides corresponding to promising recognition sites such as the RRKRK (SEQ ID NO: 10) motif were synthesized in lengths ranging from six to

thirty five residues, as shown in Table 1, below. Polypeptides corresponding to both the ENOS and NNOS insertions were selected for evaluation. In addition to constructs based on the major insertions, polypeptides corresponding to the neighboring $\alpha>\beta$ loops in all three isoforms were synthesized, because of the possibility that these loops form a significant part of the binding site for the insertion in NOS. This possibility was suggested both by their proximity to the insertion and by their negative charge.

TABLE 1 Synthetic Peptides

| Peptide | Der.[1] | Sequence | SEQ ID NO. |
|---|---|---|---|
| BO58-01 | h ENOS | AVDTRLEELGGERT | 21 |
| BO58-02 | NNOS | AVDTLLEELGGERT | 22 |
| BO58-03 | m INOS | DIDQKLSHLGASQT | 23 |
| BO58-04 | b ENOS | DDVVSLEHET | 24 |
| BO58-05 | r NNOS | DIVHLEHES | 25 |
| BO58-06 | m INOS | KASTLEEEQ | 26 |
| BO58-07 | b ENOS | WRRKRK | 12 |
| BO58-08 | b ENOS | SSWRRKRKESS | 13 |
| BO58-09 | h NNOS | QEERKSYKVRF | 16 |
| BO58-10 | h NNOS | RPEQHKSYKIRF | 17 |
| BO58-11 | r NNOS | SDSRKSSGDGPDLR | 18 |
| JX2 | b ENOS | SSPRPEQHKSYKIRFNSVSCSDPLVSSWRRKRK | 14 |
| JX3 | b ENOS | QHKSYKIRFNSVSCSDPLVSSWRRKRKE | 15 |
| JX4 | h NNOS | QEERKSYKVRFNSVSSYSDSQKSSGDGPDL | 19 |
| PEP1 | | RPEQHKSYKIRF | 27 |
| PEP2 | | QEERKSYKVRFNSVSSYSDSRKSSGDGPDL | 28 |

1 Derivation: h = human; m = mouse; b = bovine and r = rat.

JX series polypeptides are approximately 30-mers derived from ENOS and NNOS inhibitory polypeptides. BO58 series are shorter (6-mers to 15-mers) peptides derived from flanking loops or from shorter segments of inhibitory polypeptides. CAM binding data represents counts from radiolabeled CAM trapped on a membrane by NNOS adhesion. INOS activity was assayed by measuring oxidation of ferrous myoglobin by product NO. Mg/ml concentrations of larger JX series polypeptides were three times higher than others to keep molar concentrations comparable in experiments. The results of experiments to determine the effects of the synthetic polypeptides on NOS activity are shown in Table 2, below.

TABLE 2 CAM Binding and INOS Inhibition Data

| Peptide | NNOS CAM Binding | INOS Activity 0.1 mg/ml | INOS activity 0.3 mg/ml | INOS Activity 1.0 mg/ml |
|---|---|---|---|---|
| None | 774 ± 40 | 14.9 ± .56 | 12.3 ± .19 | 13.0 ± .23 |
| BO58-01 | 986 ± 58 | 15.0 ± .73 | | |
| BO58-02 | 953 ± 80 | 15.1 ± .03 | | |
| BO58-03 | 863 ± 19 | 14.4 ± .27 | | |
| BO58-04 | 903 ± 57 | 15.8 ± .73 | 14.9 ± .56 | |
| BO58-05 | 952 ± 52 | 14.6 ± .03 | | |
| BO58-06 | 951 ± 100 | 15.3 ± .32 | | |
| BO58-07 | 1749 ± 40 | 13.6 ± .31 | | |
| BO58-08 | 152 ± 26 | 14.5 ± .70 | | |
| BO58-09 | 686 ± 43 | 14.7 ± .28 | | |
| BO58-10 | 1339 ± 49 | 14.6 ± .02 | | |
| BO58-11 | 908 ± 56 | 14.7 ± .94 | | |
| JX2 | 1356 ± 46 | 13.1 ± .11 | 7.7 ± .11 | 3.2 ± .11 |
| JX3 | 674 ± 100 | 12.9 ± .32 | 7.9 ± .11 | 2.9 ± .32 |
| JX4 | 659 ± 41 | 14.1 ± .57 | 10.4 ± .57 | 8.4 ± .57 |

Several of the polypeptides based on ENOS insertions proved capable of inhibiting INOS activity. A 50% inhibition of INOS was observed within minutes after adding the polypeptides. Since the "off" constant of CAM is many orders of magnitude longer than this time frame, the result suggests that inhibition can be obtained without CAM displacement. If inhibition were dependent on simple competition between CAM and the inhibitor, it would be necessary to wait hours or days for CAM to fall off and be replaced; therefore, a ternary complex must form at least transiently. While it is not possible currently to completely eliminate the possibility that CAM is rapidly displaced from the ternary complex, the fact that, in the absence of peptide boiling, it does not completely displace CAM from INOS, suggests that the ternary complex is the inhibited species.

The most effective inhibitory polypeptides contain the motif RRKRK (SEQ ID NO: 10) from the ENOS insertion. Partial inhibition of INOS could also be obtained with NNOS-based polypeptides.

A second experiment confirmed these results. NOS activity measurements were performed using recombinant rat NNOS (purified from overexpressing HEK293 cells), recombinant bovine ENOS (purified from overexpressing E. coli) or native INOS (from immunostimulant-activated rat aortic smooth muscle cells). Activity was determined with 1-20 pmole of NOS using a kinetic 96-well microtiter plate assay based on the kinetics of NADPH consumption (NNOS and ENOS) or $Fe^{2+}$-myoglobin oxidation. Radioligand binding was performed after incubation of 1-2 pmole of NOS for 15 minutes at 23°C with either Bolton-Hunter labelled [125I]-calmodulin (1 nM) or [3H]-Nω-nitro-L-arginine (200 pM) and the indicated peptides. Results are shown in Table 3. Values are means ± SEM of triplicate determinations.

TABLE 3 Effect of Peptides on NOS Activity and Ligand Binding

| Peptide | μg/ml (μM) | NOS Activity (% of control) | | | Ligand binding to NOS | |
|---|---|---|---|---|---|---|
| | | NNOS | ENOS | INOS | [$^3$H]-NHA | [$^{125}$I]-CAM |
| BO58-07 | 100 (107.0) | 11.0 ± 3.3 | 24.0 ± 2.8 | 91.1 ± 5.7 | 91.7 ± 5.1 | 6.7 ± 3.6 |
| BO58-08 | 100 (71.2) | 19.1 ± 0.9 | 27.7 ± 2.0 | 92.1 ± 1.3 | 120.7 ± 12.7 | 0.0 ± 0.5 |
| BO58-09 | 100 (68.1) | 102.0 ± 2.1 | 93.6 ± 2.3 | 101.1 ± 3.7 | 118.9 ± 4.1 | 82.7 ± 1.1 |
| BO58-10 | 100 (63.0) | 54.5 ± 1.8 | 80.5 ± 2.0 | 99.7 ± 5.7 | 115.0 ± 8.6 | 24.7 ± 2.2 |
| BO58-11 | 100 (72.0) | 98.4 ± 1.9 | 102.6 ± 4.1 | 99.7 ± 2.8 | 119.2 ± 3.4 | 89.2 ± 6.2 |
| JX2 | 300 (76.0) | 30.4 ± 1.7 | 57.2 ± 7.5 | 62.2 ± 2.8 | 116.3 ± 1.9 | 0.0 ± 7.4 |
| JX3 | 300 (87.7) | 28.2 ± 0.9 | 40.2 ± 4.0 | 64.3 ± 3.2 | 122.4 ± 1.0 | 0.0 ± 4.8 |
| JX4 | 300 (84.8) | 103.0 ± 1.3 | 80.2 ± 3.8 | 84.1 ± 3.5 | 95.7 ± 4.7 | 82.7 ± 1.1 |

EXAMPLE 3 Effects on Calmodulin Binding

In addition to the inhibition of enzyme activity, it is possible to modulate CAM binding to NOS with synthetic homologs of the control site elements. The relationship between enzyme inhibition and CAM binding in the presence of control site elements can be complex. In other systems, antibodies to inhibitory elements sometimes induce partial CAM independent activation, while preventing CAM dependent activation. It is important to remember that CAM binding is only a means to displace the inhibitor.

The results of the experiments not only confirmed the function of the major FMN module insertion as the inhibitory polypeptide, but suggested a few details of the switching mechanism. While a number of the polypeptides could modulate CAM binding, the series containing the RRKRK (SEQ ID NO: 10) motif was the most instructive. The peptide with three residues following this motif (RRKESS, SEQ ID NO: 10) was a potent CAM antagonist with ENOS and NNOS. CAM binding was decreased almost to background levels, with effects seen at the 10 uM level.

Polypeptides which terminated at the RRKRK (SEQ ID NO: 10) motif, including good inhibitors, were promoters of CAM binding. One polypeptide which had a single amino acid after this motif had no significant effect on CAM binding. Polypeptides based on the flanking loop regions had no significant effect on INOS activity, but tended to weakly promote the binding of CAM to constitutive NOS.

EXAMPLE 4 Mechanism of NOS Control

The results presented here provide powerful evidence that the major insertion in the FMN binding module is the inhibitory polypeptide of constitutive NOS, and that its absence in INOS accounts for the lack of sensitivity of INOS to calcium, and, in part, for its very tight binding of CAM. It appears that INOS has developed from an

ancestral constitutive NOS-like protein by loss of the inhibitory peptide. The CAM binding site in INOS and constitutive NOS is apparently related to a similar basic region near the N terminal of P450 reductase, and may have developed from such a region in a common ancestral protein.

The inhibition of INOS by synthetic analogs of the constitutive NOS inhibitory polypeptides is related to the ability of the synthetic polypeptides to modulate CAM binding, but does not have the simple direct relationship expected if the mechanism of peptide inhibitor action was through CAM displacement. The reverse appears to be true: inhibition/activation of NOS at this site is driven by the occupancy of key sites by the inhibitory polypeptide, and CAM binding acts to modify the binding of the intrinsic inhibitory segment to a site or sites nearby on the surface of the enzyme.

It is not necessary to displace CAM in order to inhibit the enzyme at the control site. The data suggest that the binding domain of the inhibitory peptide has several regions. There is at least one recognition site which binds the RRKRK (SEQ ID NO: 10) motif, and there is indication of a second such site which recognizes sequences such as EERKSYKVRF (SEQ ID NO: --) and EQHKSYKIRF (SEQ ID NO: --) which occur in the N terminal half of the ENOS and NNOS insertions; peptides which lack RRKRK (SEQ ID NO: 10) but contain these sequences can be inhibitors and/or CAM binding modulators.

Ability to bind to this recognition site does not automatically confer either inhibitory character or the ability to prevent CAM binding. It is apparent that CAM binding is strongly inhibited by peptides with a short extension after the RRKRK motif; a three residue extension produced a peptide which reduced CAM binding to near background levels, while even a single residue produced a small decrease. The ability of the two polypeptides which

ended in the RRKRK motif to potentiate CAM binding strongly suggests that a region of overlap between the CAM binding site and the peptide binding site exists, in which the overlap occurs between bound CAM and residues towards the C terminal from RRKRK (SEQ ID NO: 10). In the intrinsic peptide other residues may contribute to the overlap, since it is both larger and more conformationally constrained than the synthetic analogs used here as probes.

EQUIVALENTS

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended claims. Those skilled in the art will recognize or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described specifically herein. Such equivalents are intended to be encompassed in the scope of the claims.

CLAIMS

What is claimed is:

1. An agent which inhibits nitric oxide synthase by blocking calmodulin activation of the nitric oxide synthase.

2. The agent of Claim 1, which specifically inhibits a constitutive nitric oxide synthase.

3. The agent of Claim 2, which specifically inhibits endothelial nitric oxide synthase.

4. The agent of Claim 2, which specifically inhibits neuronal nitric oxide synthase.

5. The agent of Claim 1, which specifically inhibits inducible nitric oxide synthase.

6. A nitric oxide synthase inhibitor peptide, comprising at least two positively charged amino acids selected from the group consisting of: arginine, histidine, lysine, and ornithine.

7. The peptide of Claim 6, comprising the amino acid sequence RRKRK (SEQ ID NO. 10).

8. The peptide of Claim 7, comprising the amino acid sequence of SEQ ID NO. 1 or an inhibitory fragment thereof.

9. The peptide of Claim 7, wherein the peptide binds to a site spatially adjacent to the calmodulin binding site of endothelial nitric oxide synthase.

10. The peptide of Claim 7, comprising the amino acid sequence of SEQ ID NO. 1 or a fragment or derivative thereof, wherein the derivative has one or more amino acids deleted or inserted, or has one or more conservative substitutions.

11. The peptide of Claim 7, wherein the peptide has an amino acid sequence selected from the group consisting of: WRRKRK (SEQ ID NO. 12); SSWRRKRKESS (SEQ ID NO. 13); SSPRPEQHKSYKIRFNSVSCSDPLVSSWRRKRK (SEQ ID NO. 14); and QHKSYKIRFNSVSCSDPLVSSWRRKRKE (SEQ ID NO. 15).

12. A nitric oxide synthase inhibitor peptide comprising the amino acid sequence of SEQ ID NO. 2 or a fragment or derivative thereof, wherein the derivative has one or more amino acids deleted or inserted, or has one or more conservative substitutions.

13. The peptide of Claim 12, wherein the peptide binds to a site spatially adjacent to the calmodulin binding site of neuronal nitric oxide synthase.

14. The peptide of Claim 12, wherein the peptide has an amino acid sequence selected from the group consisting of: QEERKSYKVRF (SEQ ID NO. 16), RPEQHKSYKIRF (SEQ ID NO. 17), SDSRKSSGDGPDLR (SEQ ID NO. 18), and QEERKSYKVRFNSVSSYSDSQKSSGDGPDL (SEQ ID NO. 19).

15. A nitric oxide synthase inhibitor peptide comprising the amino acid sequence of SEQ ID NO. 2, or a fragment thereof.

16. An activator of endothelial nitric oxide synthase which antagonizes autoinhibition by a peptide region of endothelial nitric oxide synthase, wherein the region is between about amino acids 590-650 of endothelial nitric oxide synthase.

17. An antibody which binds to one or more amino acids between about amino acids 590-650 of endothelial nitric oxide synthase.

18. The antibody of Claim 17, wherein the antibody activates endothelial nitric oxide synthase.

19. A constitutive nitric oxide synthase activator peptide comprising an amino acid sequence selected from the group consisting of: SEQ ID NO. 4, SEQ ID NO. 5, SEQ ID NO. 6, SEQ ID NO. 7, SEQ ID NO. 8, SEQ ID NO. 9, and activating fragments and derivatives thereof.

20. An activator of neuronal nitric oxide synthase which antagonizes autoinhibition by a peptide region of neuronal nitric oxide synthase, wherein the region is between about amino acids 820-880 of neuronal nitric oxide synthase.

21. An antibody which binds to one or more amino acids between about amino acids 820-880 of neuronal nitric oxide synthase.

22. The antibody of Claim 21 wherein the antibody activates neuronal nitric oxide synthase.

23. An antibody which binds to one or more amino acids in a peptide having an amino acid sequence selected from the group consisting of: SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4, SEQ ID NO. 5, SEQ ID NO. 6, SEQ ID NO. 7, SEQ ID NO. 8, and SEQ ID NO. 9.

24. The antibody of Claim 23, wherein the antibody is a monoclonal antibody or a fragment thereof.

25. An isolated nucleic acid encoding the antibody of Claim 24.

26. A method of inhibiting a nitric oxide synthase, comprising contacting the nitric oxide synthase with an effective amount of an agent of Claim 1.

27. A method of inhibiting endothelial nitric oxide synthase, comprising contacting the endothelial nitric oxide synthase with an effective amount of an agent of Claim 3.

28. A method of inhibiting neuronal nitric oxide synthase, comprising contacting the neuronal nitric oxide synthase with an effective amount of an agent of Claim 4.

29. A method of inhibiting a constitutive nitric oxide synthase, comprising contacting the nitric oxide synthase with an effective amount of an agent of Claim 7.

30. A method of inhibiting a constitutive nitric oxide synthase, comprising contacting the nitric oxide synthase with an effective amount of an agent of Claim 8.

31. A method of activating endothelial nitric oxide synthase, comprising contacting the endothelial nitric oxide synthase with an effective amount of an agent of Claim 16.

32. A method of activating endothelial nitric oxide synthase, comprising contacting the endothelial nitric oxide synthase with an effective amount of an agent of Claim 19.

33. A method of activating neuronal nitric oxide synthase, comprising contacting the neuronal nitric oxide synthase with an effective amount of an agent of Claim 20.

34. An agent which inhibits inducible nitric oxide synthase by blocking electron transfer from NADPH to an active site of nitric oxide synthase.

35. The agent of Claim 34, wherein the nitric oxide synthase is inducible nitric oxide synthase.

36. The agent of Claim 34, which binds to a site spatially adjacent to the calmodulin binding site of inducible nitric oxide synthase.

37. The agent of Claim 36, wherein the agent binds to one or more amino acids between about amino acids 600-615 of inducible nitric oxide synthase.

38. An activator of endothelial nitric oxide synthase which binds to one or more amino acids in a sequence selected from the group consisting of: SEQ ID NO. 23 and SEQ ID NO. 24.

39. An activator of inducible nitric oxide synthase which binds to one or more amino acids in a sequence selected from the group consisting of: SEQ ID NO. 3, SEQ ID NO. 22 and SEQ ID NO. 25.

40. An antibody which binds to one or more amino acids between about amino acids 600-615 of inducible nitric oxide synthase.

41. The antibody of Claim 40, wherein the antibody inhibits inducible nitric oxide synthase.

42. The antibody of Claim 40, wherein the antibody activates inducible nitric oxide synthase.

43. A method of treating a disease modulated by the production of nitric oxide by endothelial nitric oxide synthase in a mammal, comprising administering to the mammal an effective amount of an agent of Claim 3.

44. A method of treating a disease modulated by the production of nitric oxide by neuronal nitric oxide synthase in a mammal, comprising administering to the mammal an effective amount of an agent of Claim 4.

45. A method of treating a disease modulated by the production of nitric oxide by a constitutive nitric oxide synthase in a mammal, comprising administering to the mammal an effective amount of an agent of Claim 2.

46. A method of treating a disease modulated by the production of nitric oxide by a constitutive nitric oxide synthase in a mammal, comprising administering to the mammal an effective amount of an agent of Claim 6.

47. A method of treating a disease modulated by the production of nitric oxide by a constitutive nitric oxide synthase in a mammal, comprising administering to the mammal an effective amount of an agent of Claim 8.

48. A method of treating a disease modulated by production of nitric oxide by endothelial nitric oxide synthase in a mammal, comprising administering to the mammal an effective amount of an agent of Claim 16.

49. A method of treating a disease modulated by production of nitric oxide by endothelial nitric oxide synthase in a mammal, comprising administering to the mammal an effective amount of an agent of Claim 19.

50. A method of treating a disease modulated by production of nitric oxide by neuronal nitric oxide synthase in a mammal, comprising administering to the mammal an effective amount of an agent of Claim 20.

51. A method for identifying an agent that modulates activity of a nitric oxide synthase, comprising assaying the ability of the agent to modulate calmodulin activation of the nitric oxide synthase.

52. The method of Claim 51, wherein the nitric oxide synthase is selected from the group consisting of: endothelial nitric oxide synthase, neuronal nitric oxide synthase, and inducible nitric oxide synthase.

53. The method of Claim 51, wherein the agent inhibits the activity of the nitric oxide synthase.

54. A method for identifying agents which modulate activity of a constitutive nitric oxide synthase, comprising assaying the ability of the agent to modulate autoinhibition of the nitric oxide synthase.

55. The method of Claim 54, wherein the constitutive nitric oxide synthase is selected from the group consisting of: endothelial nitric oxide synthase and neuronal nitric oxide synthase.

56. A method for identifying an agent that modulates activity of a nitric oxide synthase, comprising assaying the ability of the agent to block electron transfer from NADPH to an active site of the nitric oxide synthase.

57. An isolated nucleic acid encoding the peptide of Claim 6 or a complement thereof.

58. An isolated nucleic acid encoding the peptide of Claim 12 or a complement thereof.

59. An isolated nucleic acid encoding the peptide of Claim 19 or a complement thereof.

**INITIAL FMN BINDING REGION**

| | | |
|---|---|---|
| NCPR_HUMAN | ESSFVEKMKK TGRNIIVFYG SQTGTAEEFA NRLSKD.AHR YGMRGMSADP | 115 |
| NOSE_BOVIN | GTLMAKRV.. ...KATILYA SETGRAQSYA QQLGRLFRKA FDPRVLCMD. | 556 |
| NOSB_RAT | GQAMAKRV.. ...KATILYA TETGKSQAYA KTLCEIFKHA FDAKAMSME. | 789 |
| NOSM_MOUSE | RKVMASRV.. ...RATVLFA TETGKSEALA RDLATLFSYA FNTKVVCMD. | 567 |
| FLAV_ECOLI | ..... ..AITGIFFG SDTGNTENIA KMIQKQL.GK .D.VADVHDI | 35 |
| FLAV_DESVH | ..... .MPKALIVYG STTGNTEYTA ETIARELADA .GYEVDSRDA | 38 |

**FLANKING LOOP SECOND FMN SITE**

| | | |
|---|---|---|
| NCPR_HUMAN | EEYDLADLSS LPEIDNALVV FCMATYGEGD PTDNAQDFYD WL.QE..... | 159 |
| NOSE_BOVIN | .EYDVVSL.. ...EHETLVL VVTSTFGNGD PPENGESFAA AL.MEMSGPY | 599 |
| NOSB_RAT | .EYDIVHL.. ...EHEALVL VVTSTFGNGD PPENGEKFGC AL.MEMRHP. | 831 |
| NOSM_MOUSE | .QYKASTL.. ...EEEQLLL VVTSTFGNGD CPSNGQTLKK SLFML..... | 606 |
| FLAV_ECOLI | AKSSKEDL.. ...EAYDILL LGIPTWYYGE ....AQCDWD DF.FP..... | 70 |
| FLAV_DESVH | ASVEAGGLF. ...EGFDLVL LGCSTWGDDS IE..LQDDFI PL.FD..... | 76 |

**LOCATION OF REGULATORY LOOP INSERT**

| | | |
|---|---|---|
| NCPR_HUMAN | ..... ..... ..... ..... TDVDLSGVKF | 169 |
| NOSE_BOVIN | NSSPRPEQHK SYKIRFNSVS CSDPLVSSWR RKRKESSNTD SAGAGTLRFL | 649 |
| NOSB_RAT | NS..VQEERK SYKVRFNSVS SYSDSRKSSG DGPDLRDNFE STGPLANVRF | 879 |
| NOSM_MOUSE | ..... ..... ..... ..... .RELNHTFRY | 615 |
| FLAV_ECOLI | ..... ..... ..... .....TL EEIDFNGKLV | 82 |
| FLAV_DESVH | ..... ..... ..... .....SL EETGAQGRKV | 88 |

**THIRD FMN SITE FLANKING LOOP**

| | | |
|---|---|---|
| NCPR_HUMAN | AVFGLGNKT. Y.EHFNAMGK YVDKRLEQLG AQRI | 201 |
| NOSE_BOVIN | CVFGLGSRA. Y.PHFCAFA. AVDTRLEELG GERL | 680 |
| NOSB_RAT | SVFGLGSRA. Y.PHFCAFGH AVDTLLEELG GERI | 911 |
| NOSM_MOUSE | AVFGLGSSM. Y.PQFCAFAH DIDQKLSHLG ASQL | 647 |
| FLAV_ECOLI | ALFGCGDQED YAEYFCDALG TIRDIIEPRG ATIV | 116 |
| FLAV_DESVH | ACFGCGDSS. Y.EYFCGAVD AIEEKLKNLG AEIV | 120 |

FIG. 1

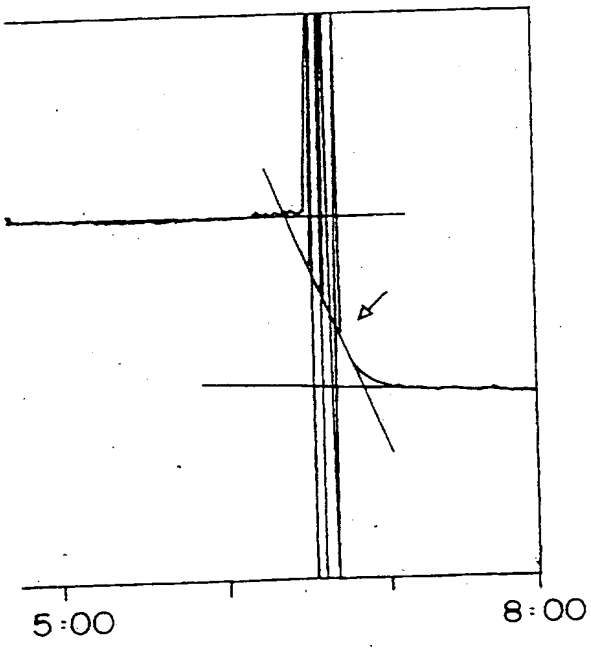


FIG. 2A

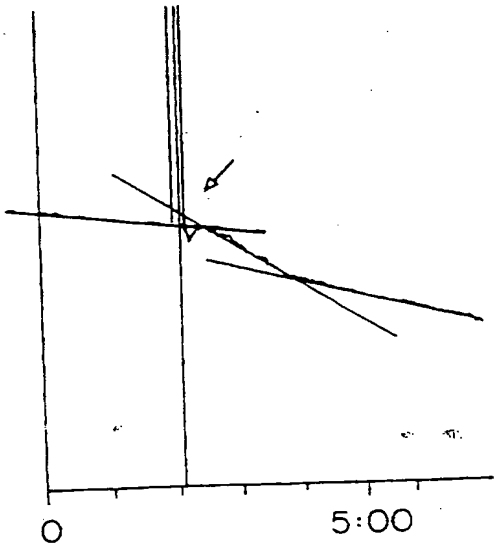


FIG. 2B

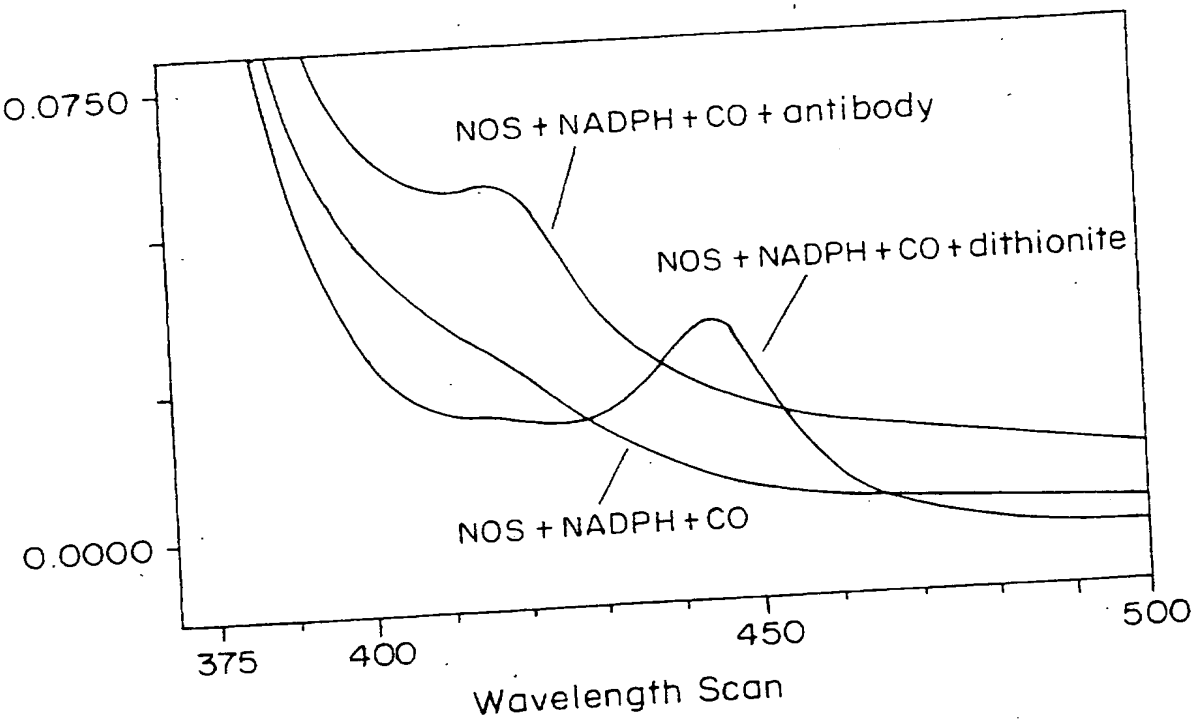


FIG. 2C

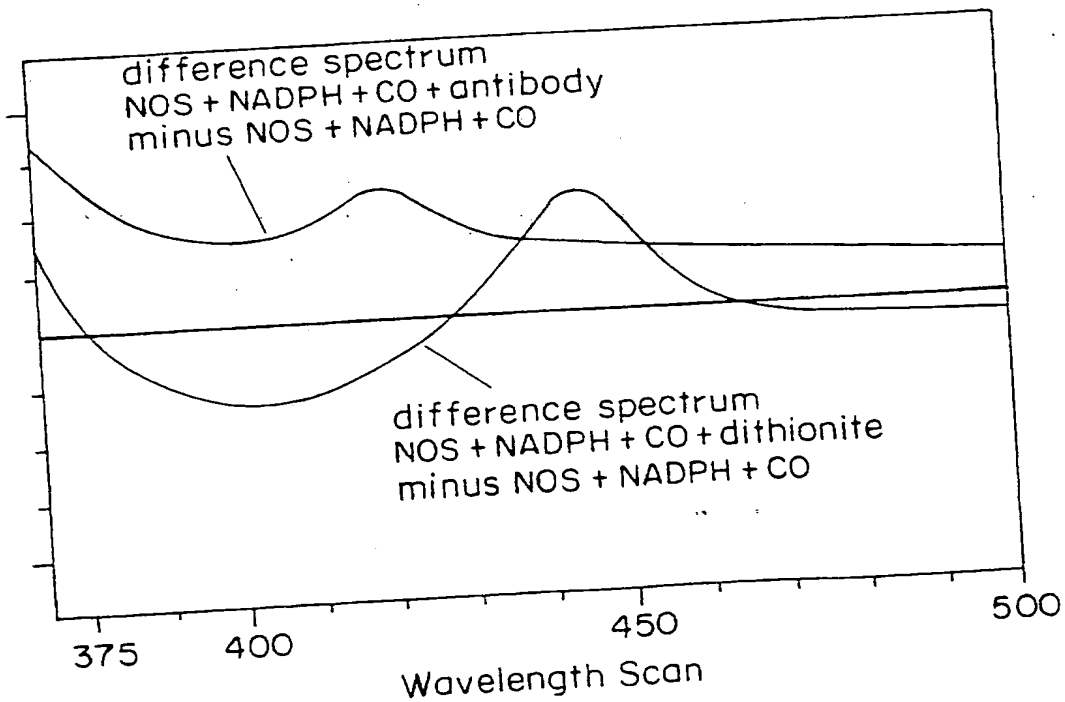


FIG. 2D

# INTERNATIONAL SEARCH REPORT

Interr nal Application No
PCT/US 97/12568

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/53 C12N15/12 C12N15/13 C07K14/47 C12N9/02
C07K7/06 C07K7/08 C07K16/18 C07K16/40 A61K38/43
C12Q1/26

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | MIRIAM PALACIOS: "Chlorpromazine inhibits both the constitutive nitric oxide synthase and the induction of nitric oxide synthase after LPS challenge" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 196, no. 1, 15 October 1993, ORLANDO, FL US, pages 280-286, XP002046701 see abstract see page 282, last paragraph - page 283, paragraph 1 --- -/-- | 1,26, 51-53 |

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 13 November 1997 | 09.12.97 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016 | Montero Lopez, B |

Form PCT/ISA/210 (second sheet) (July 1992)

# INTERNATIONAL SEARCH REPORT

Inter ional Application No
PCT/US 97/12568

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WOLFF D J ET AL: "CALMODULIN-DEPENDENT NITRIC-OXIDE SYNTHASE MECHANISM OF INHIBITION BY IMIDAZOLE AND PHENYLIMIDAZOLES" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 268, no. 13, 5 May 1993, pages 9425-9429, XP000652112 | 56 |
| A | see abstract see page 9428, left-hand column, paragraph 3 - page 9429, left-hand column, paragraph 1 | 34-37 |
| | --- | |
| A | MASAKI NAKANE ET AL: "NOVEL POTENT AND SELECTIVE INHIBITORS OF INDUCIBLE NITRIC OXIDE SYNTHASE" MOLECULAR PHARMACOLOGY, vol. 47, no. 4, 1 April 1995, pages 831-834, XP000566159 see abstract see page 832, right-hand column, paragraph 1 - page 834, left-hand column, last paragraph | 1-59 |
| | --- | |
| P,X | WATANABE, YASUO ET AL: "Identification of a specific amino acid cluster in the calmodulin -binding domain of the neuronal nitric oxide synthase" FEBS LETT. (1997), 403(1), 75-78 CODEN: FEBLAL;ISSN: 0014-5793, 1997, XP002046702 see abstract see page 76, left-hand column, last paragraph - page 77, right-hand column, paragraph 1 | 1,2,4,6, 26,28, 44-46, 51-53,57 |
| | --- | |
| P,X | MAYER, BERND ET AL: "A synthetic peptide corresponding to the putative dihydrofolate reductase domain of nitric oxide synthase inhibits uncoupled NADPH oxidation" NITRIC OXIDE (1997), 1(1), 50-55 CODEN: NIOXF5;ISSN: 1089-8603, 1997, XP002046703 see abstract see page 51, right-hand column, last paragraph - page 53, left-hand column, paragraph 2 | 34,56 |
| | ----- | |

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

# INTERNATIONAL SEARCH REPORT

.rnational application No.
PCT/US 97/12568

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 97/12568

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Remark : Although claims 26-33, as far as concerning an in-vivo method, and claims 43-50 are directed to a method of treatment of the human/animal body , the search has been carried out and based on the alleged effects of the compounds.